THE INFLUENCE OF DIPHTHERIA TOXOID ON THE HISTAMINE CONTENT OF THE LUNG AND THE LETHAL DOSE OF HISTAMINE

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It has been shown previously that past experience of a histamine liberating agent affects the ease of release of histamine from the lung by another agent and also increases the tolerance of the animal to the toxic effect of histamine (Trethewie and Day, 1948). It has further been shown that animals treated with "Serogan" and gonadal hormone also show an increase in the LD_{50} for histamine (Trethewie and Gaffney, 1949, 1950). In this paper we have investigated the effect of diphtheria toxoid on the toxicity of histamine and the histamine level in the lungs of injected animals.

METHOD.

Selection of the animals.

Forty-three guinea-pigs were obtained from the same breeding source. They were randomized into two groups. The control animals were tagged on the left ears and the animals to be treated on the right ears. When the guinea-pigs were two weeks of age they were given their first injection: 0·1 ml. saline to the controls and 0·1 ml. formolized diphtheria toxoid (C.S.L.) to the treated animals. Injections were made subcutaneously. In all, five injections were made at intervals of one week.

Histamine assay.

When the animals were seven weeks old, the lungs of twenty-two of them (thirteen treated and nine untreated animals) were perfused and the content of histamine was determined after squeezing the lung between filter paper and weighing. The lung was extracted with silica in Tyrode and boiled (Feldberg and Kellaway, 1937). The histamine was estimated as histamine acid phosphate on the isolated jejunum of the guinea-pig suspended in Tyrode.

In a number of experiments (Trethewie, 1947) assays have been made of the histamine content of the lungs as histamine acid phosphate. It might be that such a procedure is unsound if graded doses of phosphate do not produce the same change in response produced by graded doses of histamine in the form it is in the cell, e.g. assay of 1 mg. lung may then yield the figure 38 μg. histamine/gm. while 2 mg. may yield only 60 μg. histamine/gm. Now it has been shown already (Trethewie, 1938) that assay of the lung by the method of Best (1929),

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gives the same histamine figure as does simple boiling when the comparison is made with dihydrochloride. Evidently then the dihydrochloride does produce graded responses in parallel with the responses to histamine as in the cell since the histamine was converted to dihydrochloride by Best's method. A comparison has therefore been made between the response of the gut to acid-phosphate and dihydrochloride to determine this point.

The assays have been carried out on different days. The acid-phosphate was kept for some time in a vacuum desiccator (caustic dehydration) and the rather hygroscopic dihydrochloride, also kept in the desiccator, was a fresh tube just opened for use.

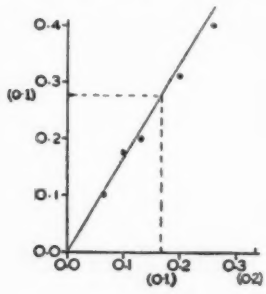

Fig. 1. Comparison of activity of histamine acid-phosphate and histamine dihydrochloride; abscissa: dose of dihydrochloride (μg.); ordinate: dose of acid-phosphate (μg.) equivalent to corresponding response to dihydrochloride. Figures in brackets indicate content of base in salts.

In some of the tests, various doses of histamine dihydrochloride were added to the bath until a certain dose gave the same response as a given dose of histamine acid-phosphate. In the other tests, various doses of histamine acid phosphate were added to the bath until a certain dose gave the same response repeatedly to a given dose of histamine dihydrochloride. When the salt used was changed, the gut was tested repeatedly thereafter with the original test dose to ensure that the gut had returned to its original sensitivity and the salt used for comparison was not applied again until the gut responses were constant. The results were as follows:

In the first test 0·26 μg. histamine dihydrochloride equalled 0·4 μg. histamine acid-phosphate in its response as assayed on the guinea-pig jejunum and 0·13 μg. dihydrochloride gave the same contraction as 0·2 μg. acid-phosphate.

In the second test, 0·3 μg. acid phosphate gave a response corresponding to 0·2 μg. dihydrochloride and 0·175 μg. acid phosphate to 0·1 μg. dihydrochloride.

In the next test, 0·065 μg. dihydrochloride equalled 0·1 μg. acid-phosphate in its response on the gut and 0·13 μg. dihydrochloride equalled that to 0·2 μg. acid-phosphate.

By graphing the activity of the gut to the phosphate and the dihydrochloride (Fig. 1) a reasonably linear result was obtained so that presumably our assay of the lungs simply boiled is sound within the range of concentration tested. The figures in brackets (Fig. 1) indicate the amount of base in each salt (estimated after the experiment was completed). The individual estimates for the dihydrochloride average 4 p.c. lower than would be accounted for by the content of base and in view of the slope and the detailed assaying procedure outlined above (to secure accuracy), this may be due to the fact that the dihydrochloride contains some water which we did not remove in our vacuum desiccator.

Toxicity of histamine.

Eight treated and nine untreated animals were injected subcutaneously on an uninjured part of the abdomen with 4·8 mg./kg. histamine acid-phosphate (10 mg./ml.). The death times were noted in minutes and the survivors were observed for 48 hours and then they were sacrificed.

EXPERIMENTAL.

The influence of diphtheria toxoid on the histamine content of the lungs.

The histamine content of the lungs was estimated in thirteen of the treated group and nine of the controls. The histamine content of the lungs of the treated group varied from 37–230 μg./gm. with an average of 99. In the control group the histamine contents varied

from 31–135 μg./gm. with an average of 66 (Table 1). Since we also determined the weight of the lungs, we were able to estimate the histamine content in the whole lungs. This was 104–736 μg. in the treated series (av. 315) and 128–336 μg. (av. 221) in the control group.

TABLE 1.

Histamine content (H.) of the lung of treated and control guinea-pigs.

No. animal	μg./gm. H. in lung	Weight lung (gm.)	Total H. in lung (μg.)	Weight animal (gm.)	H. in μg./gm. animal
Treated:					
23	111	3·549	393·9		
24	70	3·215	225·1		
26	37	3·370	124·7		
28	100	3·103	310·3		
29	100	3·845	384·5	432	0·890
30	46	2·267	104·3	313	0·333
31	46	3·006	138·3	358	0·386
32	125	3·053	381·6	469	0·814
35	150	3·137	470·6	399	1·180
39	100	3·510	351·0	446	0·801
40	114	2·804	319·7	405	0·789
41	230	3·201	736·2	402	1·831
43	56	2·700	151·2	305	0·496
Average	99		315		
Control:					
20	62	3·512	217·6	473	0·460
21	31	4·134	128·2	479	0·268
22	65	2·618	170·2		
25	40	3·760	150·4		
27	40	4·320	184·8		
33	77	3·526	271·5	473	0·574
34	50	4·953	247·7	445	0·557
36	125	2·686	335·8	387	0·846
37	100	2·810	281·0	436	0·644
Average	66		221		

TABLE 2.

	Above average	Below average
Treated	8	5
Untreated	4	12

By statistical analysis it was found that the difference in the histamine content of the lungs (43 p.c.) of the treated and untreated groups was not significant. The difference in the concentrations of histamine in μg./gm., (50 p.c.), was not significant either. We then increased the number of animals in the control group by including estimates from a further seven animals of the same size, from the same source. These animals had an average histamine

content of 60 μg./gm., and an average total histamine content of 183 μg. Statistical analysis shows that the difference in histamine content (μg./gm.) in this larger series is significant (t, 2·4 : $p < 0·05$); also the difference in total histamine content (μg.) is significant (t, 2·4 : $p < 0·05$). When the average total content of histamine in the two groups is averaged, it is found that in the treated group, five are in the "below average" group, and eight are within the "above average" group. In the untreated group twelve are in the lower and four in the upper group (Table 2). By statistical analysis this is found to give p nearly equal 0·05, which is on the level of significance.

Toxicity of histamine.

When 4·8 mg./kg. histamine were injected, it was found that all of the test animals survived, whereas only four of the control animals survived and five of them died. By statistical analysis it was found that the difference in the mortality is significant (p, 0·02).

DISCUSSION.

In investigations on the histamine content of the lungs of guinea-pigs it has been found that the content at birth is 4–5 times that at 8 weeks of age (Trethewie, 1947). Further studies showed that the injection of peptone increased significantly the ease of release of histamine by trypsin and also diminished the toxicity of histamine in the animals so injected (Trethewie and Day, 1948). The histamine content of the lungs of the treated animals was 24 p.c. greater than controls but this difference was not significant statistically though there was a significantly greater number of animals with high histamine contents among the treated series. Similar investigations with Serogan (Trethewie and Gaffney, 1949) and with gonadal hormones (Trethewie and Gaffney, 1950) showed a similar trend but more importantly a considerable increase in the tolerance of the animals to injected histamine. In the case of the former twice as much histamine was released from the perfused lungs by a standard trypsin injury compared with controls and in addition histamine inhibitor was also released from the perfused lungs of the Serogan injected animals. The studies recorded in this present paper show a statistically significant increase in the histamine content of the lungs following prior treatment of the intact animal with diphtheria toxoid (admittedly formolized). This increase was greater in magnitude than that found following injection of peptone or hormone. There was also a significant reduction in mortality of treated animals when injected subcutaneously with a dose of histamine greater than the LD_{50}. It is not inconceivable that these findings may be important in relation to a phenomenon we have termed a "non-specific immunity" effect (Trethewie, 1949).

The phenomenon of over-reaction to general infectious disease of young adults compared with the child, which has been particularly discussed in relation to the influenza pandemic of 1918–1919, may be related to the changes we have found to occur following the injection of bacterial toxin. The increased mortality of young adults in the 15–35 age group was striking in the influenza

pandemic of 1918–1919. It is also recognized that young children (except the very young) tolerate the infectious diseases more readily than the adult. This might be due to repeated exposure increasing the reactiveness to the inevitable casual infections (as we found for peptone) in the older age group and/or endocrine factors might operate. For both of these we have produced some evidence as outlined above.

On the other hand, the ease of release of histamine will determine the amount liberated and this also might be affected as is the case with peptone (Trethewie and Day, 1948). In the latter instance more histamine was liberated by a standard injury from the lungs with the higher histamine content. This increase in local reactivity may be of survival value in regard to local infections so long as the liberation of toxic products does not become generalized when such an effect is inimical. This latter effect may be counter-balanced by the reduced toxicity of histamine (as we have found with peptone, Serogan and bacterial toxin) in such cases where histamine release is important in death. If histamine release is an index of similar changes to other liberated substances this changed reactiveness may be an important phenomenon which forms a rational basis for understanding the varying impact of infectious disease on different age groups.

SUMMARY.

The histamine content of the lungs (per unit weight) is significantly raised in animals treated with diphtheria toxoid by injection.

The resistance of animals to the lethal effect of histamine (injected subcutaneously) is increased significantly in the animals treated with diphtheria toxoid when compared with controls.

REFERENCES.

Best, C. H. (1929): J. Physiol., 67, p. 256.
Feldberg, W. and Kellaway, C. H. (1937): *Ibid.*, 90, p. 257.
Trethewie, E. R. (1938): Austral. J. exp. Biol., 16, p. 225.
Trethewie, E. R. (1947): J. Immunol., 56, p. 211.
Trethewie, E. R. (1949): Med. J. Austral., I, p. 597.
Trethewie, E. R. and Day, Allan J. (1948): Austral. J. exp. Biol., 26, p. 323.
Trethewie, E. R. and Gaffney, Fay M. (1949): *Ibid.*, 27, p. 523.
Trethewie, E. R. and Gaffney, Fay M. (1950): *Ibid.*, 28, p. 589.

THE PREFORMED AMMONIA CONTENT OF SKELETAL MUSCLE AND LIVER IN EXPERIMENTAL ANURIA

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The toxicity of urea in mammals has long been a vexed question. Although most investigations seem to suggest that this substance, as such, is not toxic, the possibility still exists that a sustained high level of urea in the blood may, in accordance with the law of mass action, result in the accumulation of intermediary nitrogenous compounds which are toxic. Since it has been shown (Schmidt *et al.*, 1950) that the concentration of amino acids in the plasma remains within normal limits in renal insufficiency, it would seem reasonable to assume that in this condition the process of deamination in the liver is not suppressed. The question thus arises, whether a high blood urea level, in the presence of continued deamination of amino acids, might not lead to an accumulation of ammonium ion. The following investigation was carried out in order to examine this possibility.

Experimental method.

In ten female guinea-pigs each weighing about 500 gm. both ureters were tied. After three days all the animals showed signs of severe "uraemia." They were very weak, walked with a laboured and tremulous gait, and exhibited spontaneous twitching of the limbs and trunk. Between seventy-two and eighty hours after ligation of its ureters, each animal was killed instantaneously and small sections of liver and skeletal muscle, about 50 mg. in weight, were removed at once for the determination of their preformed ammonia content. It was considered preferable to estimate the preformed ammonia content of the tissues themselves, rather than of the blood. The free ammonia content of normal circulating blood would appear to be below the analytically determinable level (Conway, 1947), whereas the free ammonia content of resting muscle or of liver is well above this level. Owing to the fact that the ammonium ion is thus concentrated in the tissues, it seems probable that any accumulation of ammonia would be more readily detected in the tissues than in the blood.

The estimation of the preformed ammonia was carried out by the microdiffusion technique described by Conway, the sections of muscle and liver being placed directly into prepared Conway units after removal from the guinea-pig.

Two ml. of blood were also taken from each animal immediately after killing, in order to determine the blood urea level. This was estimated by the method of Van Slyke and Cullen.

Ten normal female guinea-pigs of about the same weight were used as controls.

RESULTS.

Anuric group.			Control group.		
* NH_3 in skeletal muscle	† NH_3 in liver tissue	‡ Blood urea	* NH_3 in skeletal muscle	† NH_3 in liver tissue	‡ Blood urea
0·47	0·51	155	0·56	0·53	15
0·39	0·56	162	0·41	0·40	16
0·56	0·51	172	0·33	0·46	18
0·34	0·47	190	0·65	0·57	16
0·61	0·40	185	0·51	0·58	20
0·61	0·61	186	0·54	0·50	14
0·56	0·70	190	0·44	0·63	14
0·64	0·58	149	0·61	0·60	22
0·40	0·50	170	0·52	0·54	12
0·86	0·44	200	0·40	0·59	18
Mean = 0·544	Mean = 0·528		Mean = 0·497	Mean = 0·540	

* Preformed ammonia content of skeletal muscle, expressed as mg. of ammonia nitrogen per 100 gm. of tissue.

† Preformed ammonia content of liver tissue expressed as mg. ammonia nitrogen per 100 gm. of tissue.

‡ Blood urea expressed as mg. of urea nitrogen per 100 ml.

In the case of the skeletal muscle estimations, the observed difference between the means of the two groups was 0·047, whereas the standard error of the difference was 0·17. In the case of the liver estimations, the observed difference was 0·012, the standard error of the difference 0·11.

SUMMARY.

In a group of guinea-pigs in which the ureters had been tied, signs of severe "uraemia," and marked elevation of the blood urea level, were not accompanied by any significant change in the preformed ammonia content of the liver or skeletal muscle.

REFERENCES.

Schmidt, E. G., McElvain, Norma F., Bowen, J. J. (1950): Amer. J. clin. Path., 20, p. 253.

Conway, E. J. (1947): Microdiffusion Analysis and Volumetric Error. Crosby Lockwood and Son.

STUDIES IN EXPERIMENTAL IMMUNOLOGY OF INFLUENZA

VIII. PATHOTOPIC ADJUVANTS

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Post-vaccination immunity against epidemic influenza is of two distinct types, the difference being in the distribution of antibody produced. After nasal administration of a killed virus preparation the amount of antibody demonstrable in the respiratory tract is round 5 p.c. of that contained in the total circulating plasma of the animal, or as we put it earlier, the antibody distribution coefficient is 5 (Fazekas de St.Groth and Donnelley, 1950b); if the same amount of antigen is given peritoneally or subcutaneously for instance, the distribution coefficient will be roughly 0·4, i.e. only 1/250 of the serum antibody will find its way into the bronchi.

Since specific resistance to influenza infection can be fully accounted for by the amount of antibody present on the surface of the respiratory tract (Fazekas de St.Groth and Donnelley, 1950c), nasal vaccination might seem, superficially, the method of choice. This, however, is not the case because only limited amounts of antigen can be administered by the nasal route, and the major portion of even this small dose is lost in the upper air passages. Combination of antigen-economy with distribution-efficiency was achieved by what we termed *pathotopic potentiation* of immunity. The distribution of antibody after extrarespiratory vaccination can be shifted in favour of the respiratory tract by the simultaneous nasal administration of an "adjuvant" (in the original experiments, of a heterologous type of influenza virus in itself incapable of affording any cross-protection). This change to the more advantageous distribution of antibody goes together with a significant rise in specific resistance (Fazekas de St.Groth and Donnelley, 1950d).

Since the pathotopic adjuvant did not bring about any increase in the amount of antibody present in the organism, one has to suppose that the underlying mechanism is some kind of local change allowing more serum (or perhaps only the antibody fraction of it?) to ooze out into and accumulate in the lower air passages. There is no reason why this property should be re-

[1] This work was carried out under a grant from the National Health and Medical Research Council, Canberra.

stricted to influenza viruses, and it is the aim of this paper to report on the efficacy of various substances as *pathotopic adjuvants*.

The action of influenza viruses on the respiratory tract has four main features, and accordingly four distinct groups of substances, each imitating one aspect of the virus, were tested. (1) Influenza vaccines are good antigens of submicroscopical dimensions; experiments were done therefore with another animal virus (vaccinia) and a bacterial virus (Shigella-phage C-16); (2) since influenza virus is capable of acting on the "receptors" of susceptible cells, two compounds with the same site of action, the "receptor destroying enzyme" of *V. cholerae* and the metaperiodate-ion, were tested; (3) influenza virus has a "toxic effect" on mucosal membranes by increasing their permeability, hence a number of chemical agents with similar action were included; (4) the intranasal inoculum might exert a nonspecific irritation on the lining of the respiratory tract; accordingly known irritants were given nasally together with the diluents in which influenza vaccines are usually made up.

MATERIALS AND METHODS.

Normal saline. 9·0 gm. NaCl in 1,000 ml. distilled water.

Red blood cells. Thrice washed cells of Leghorn fowls were used as a 1 p.c. suspension in normal saline. Since some of the samples to be tested contained lipid agglutinins (Fazekas de St.Groth, 1950a; Fazekas de St.Groth and Donnelley, 1950a), only lipid insensitive cells (Stone, 1946) were selected for the antibody titrations.

Influenza virus. The Melbourne strain (type A) was grown in the allantois of 11-day chick embryos. Freshly harvested fluids containing $10^{8.5}$ ID_{50}/0·05 ml. on the average were used for the experimental infection of mice. For the preparation of *vaccines* only fluids of exceptionally high haemagglutinin titre (> 1,200) were retained. To pools with an average titre of 1,400 was added 0·02 p.c. formaldehyde (= 0·05 p.c. formol), and the mixture was incubated at 37° C. overnight. The resulting preparations had retained their full haemagglutinating activity, but showed no infectivity when tested in eggs. They will be referred to as "MEL-vaccines".

Vaccinia virus. The Gillard strain was grown on the chorioallantoic membrane of 12-day chick embryos. The inoculum contained 100 times the amount of virus necessary to cause confluent pocks. The membranes, after a growth period of 42 hours, were ground with sterile alundum and suspended in 1·0 ml. of normal saline. Such preparations had an infectivity of the order of 10^7/0·05 ml.

Shigella-phage C-16. The host organism, *Shigella flexneri YS*, was grown in nutrient broth for 5 hours, and then 1·0 ml. of the bacterial culture and roughly 10^6 particles of the virus were inoculated into 50 ml. of broth buffered at pH 8·0. After an incubation of 16 hours at 37° C. the clear fluid was heated at 56° C. for half an hour to destroy surviving resistant bacteria. As determined by plating methods, the preparation contained 10^{11} viable phage particles per ml.

Receptor destroying enzyme of V. cholerae (RDE) was prepared according to the original method of Burnet and Stone (1947).

Titration of antihaemagglutinin. The small-volume technique of Fazekas de St.Groth and Donnelley (1950b) was followed. Sera and bronchial washings were collected and pre-treated as described in the paper quoted.

Assessment of immunity. The protection-test devised earlier in the course of these studies was used without modification (Fazekas de St.Groth and Donnelley 1950e). The Tables below will give the values of the experimentally determined "total lung lesions" (ΣL) following challenge with graded doses of active influenza virus, together with the difference of these (d_x) from that of a group of normal control mice. From these figures and the equivalent of a tenfold reduction in infectivity (d_{10}), as determined in the controls, the degree of specific resistance can be computed. The protection-values (P) show how many times more resistant a group of animals was than a similar group of untreated controls.

For full details of the technique and a critical discussion see the original paper.

EXPERIMENTS.

General technique.

All experiments of the present study followed a set pattern.

On the day of *immunization* the mice received an inoculum of formolized influenza virus (type A, strain Melbourne), 0·10 ml. volumes intraperitoneally. The virus content of the vaccines was kept as constant as possible for the whole series. Variations in haemagglutinin titres were less than ± 12 p.c.; the actual values will be specified in the Tables below. The compounds to be tested for their adjuvating effect were given by the respiratory route. Volumes of 0·05 ml. were instilled intranasally. If an animal received two inoculations they were given within three hours of each other, i.e. "simultaneously".

For the sake of brevity the experimental groups will be referred to by symbols, as was done in the previous papers of this series. Thus the routes of vaccination will be designated *p* and *n*, for peritoneal and nasal respectively; *A* stands for the A-type influenza virus vaccine.

An experiment in which the activity of e.g. *X*-substance was to be tested contained the following five groups: *ApXn*, the main experimental group in which *X* was given nasally to peritoneally vaccinated animals. *Ap*, the vaccine control and *ApXp*, to test the effect of *X*-substance on antibody production. *Xn*, a control to show the effect of *X*-substance on the infectibility of mice, and finally *0*, a group of untreated normal animals. Each of these groups consisted of 40 animals.

At the day of *challenging*, eleven days after vaccination, 10 mice of each group (with the exception of *0*, the negative controls) were killed, their blood collected and bronchial washings taken by the technique described under Methods. The antihaemagglutinin titre of these samples was determined individually within 24 hours. The figures in the Tables give the geometric mean titres of the groups of 10 mice.

The remaining 30 animals were divided into six sub-groups of 5, and challenged with dilutions (10^0–10^{-5}) of active MEL-virus. The group of 40 normal control mice, *0*, received inocula ranging from 10^0 to 10^{-7}. The virus used for challenging was a freshly harvested allantoic fluid preparation. It was not titrated for infectivity previously, so that minor variations from test to test were inevitable. This, however, does not influence the final results, where we are concerned only with the *relative increase* in protection between groups *Ap* and *ApXn*.

Animals dying spontaneously during the next six days were autopsied, and the degree of pulmonary consolidation noted. All survivors were sacrificed on the seventh day after challenging, and their lung lesions evaluated. The degree of resistance was assessed by the quantitative method previously described (Fazekas de St.Groth and Donnelley, 1950e).

Based on earlier experience, the dose of vaccine was so adjusted that the expected protection values should be of the order of thousand. By this means it was possible to work within the range where the accuracy of the infectivity test is such that a $\log_{10}$ 1·0 difference (tenfold difference) in protection values is statistically significant.

All experiments were repeated at least twice with different reagents, at an interval of six months. Since consistent results were obtained throughout, the Tables will give the details of the first experiment of each kind only.

Assay of viruses as pathotopic adjuvants.

The two viruses to be tested were chosen at random, mainly because they were available in the laboratory at the time of the experiments. Neither of them is a natural pathogen for the mouse, but while the virus of vaccinia is capable of growing in this host at a very slow rate, multiplication of the bacterial virus is not supported at all. However, both are excellent antigens, and their sizes are of the order of the influenza virus. This superficial similarity appeared sufficient to warrant their being tested for adjuvating activity.

The *virus of vaccinia*, in form of ground chorioallantoic membrane preparations, was diluted 1:100 in normal saline, since more concentrated suspensions caused macroscopic lesions of the lungs after nasal administration, and were found to kill mice occasionally. The dilution used in the experiments is known to elicit a satisfactory antibody response when given by the nasal route (Fenner, 1947).

TABLE 1.

Assay of vaccinia virus as pathotopic adjuvant.

Vaccination	Antibody against MEL		Infectivity test			Protection (P)
	Serum	Bronchi	ΣL	d_x	log P	
Ap	316	2·9	25·88	8·41	3·84	6,920
ApVp	352	2·7	25·14	9·15	4·18	15,100
ApVn	281	6·4	22·77	11·52	5·27	186,000
Vn	—	—	33·17	1·12	0·51	3
0 (nil)	—	—	34·29	d_{10} = 2·19	0·00	1

— = < 5 in serum and < 0·5 in bronchi.
A = influenza A vaccine (MEL-strain, formolized; titre: 1,600).
V = vaccinia virus (approx. 10^5 egg–ID_{50}/0·05 ml.).
p = peritoneally.
n = nasally.

The experimental findings summarized in Table 1 show that the virus of vaccinia when administered by the nasal route has no effect on the infectibility of normal mice with influenza virus (no difference between *Vn* and *0*). However, when the same inoculum was given to animals immunized peritoneally with an influenza vaccine, it exerted a definite potentiating effect on the resistance of this group, that is, it acted as a pathotopic adjuvant. It should be noted that the serum antibody titres are uniform in all groups receiving influenza vaccine, but the bronchial antibody content of group *ApVn* is considerably higher than that of any other group. This means, obviously, that the enhancement of protection is not the result of enhanced production of antibody

but of its accumulation in the respiratory tract, i.e. of a change in antibody distribution. The fact that vaccinia virus has no effect on antibody production becomes evident when groups *Ap* and *ApVp* are compared: there is no difference in the immunological behaviour of these two.

The *bacterial virus*, Shigella-phage C-16, was used in the form of a high titre lysate containing 10^{11} infective units per ml. Since the preparation had no untoward effects on mice, it was used in the experiments in its original concentrated form. No pathological signs were observed during the eleven days following either the nasal or the peritoneal administration of the phage suspension.

TABLE 2.

Assay of bacteriophage C-16 as pathotopic adjuvant.

Vaccination	Antibody against MEL		Infectivity test			Protection (*P*)
	Serum	Bronchi	ΣL	d_x	log *P*	
Ap	316	2·9	25·88	8·41	3·84	6,920
ApCp	339	2·9	26·02	8·27	3·78	6,040
ApCn	287	3·1	25·32	8·97	4·09	12,300
Cn	—	—	33·32	0·97	0·44	3
0 (nil)	—	—	34·29	$d_{10} =$ 2·19	0·00	1

— = < 5 in serum and < 0·5 in bronchi.
A = influenza A vaccine (MEL-strain, formolized; titre: 1,600).
C = Shigella-phage C-16 (approx. $10^{9.7}$ particles/0·05 ml.).
p = peritoneally.
n = nasally.

The results presented in Table 2 are completely negative. The phage had no effect on the susceptibility of mice to infection, or on the immune response following vaccination with killed influenza virus. Neither was there any hint of a potentiating effect in group *ApCn*. Twice repeated, the experiment gave consistently negative results. In the later tests all reagents, especially the phage, were checked before and after use and were found to possess full activity.

Such a difference between the effect of the two viruses was unexpected and puzzling. Since no obvious explanation offered itself, further experiments were devised to study more closely the behaviour of the two viruses in the animal organism. Although done at the same time, these investigations are somewhat remote from the theme of the present study, and will be reported therefore only in the next paper (Fazekas de St.Groth, 1951).

Assay of agents acting on cellular receptors.

The second group of compounds to be tested consisted of the "receptor destroying enzyme" of *V. cholerae* (RDE) and the metaperiodate-ion. Both are known to act on the surface of cells which possess "receptors" for

the influenza group of viruses, i.e. which are capable of adsorbing the virus particles.

If successful pathotopic potentiation had anything to do with the state of virus receptors on the susceptible cell, the effect of influenza virus action might be imitated by one of these two reagents. The amounts administered were so chosen that RDE should *destroy* at least 90 p.c. of receptors normally available in the respiratory tract, and IO_4-treatment should *modify* more than 90 p.c. of the receptors.

The "*receptor destroying enzyme*" preparation, of which 0·05 ml. was instilled intranasally, had a titre of 1,800 by the standard technique. It has been shown previously (Fazekas de St.Groth, 1948a) that this treatment results in progressive destruction of receptors for about eight hours. The minimum level of receptors for the MEL-strain is approximately 5 p.c. of the original. After a lag of twelve hours the number starts to rise, and attains the normal level round the sixth day. There is a slight hyperregeneration noticeable, but a fortnight after the initial treatment the process has obviously come to an end: there is no difference from the normal. In the actual experiments the state of receptors has been checked on days 1, 3, 7 and 10 after RDE-treatment; the findings were in agreement with the expectation.

TABLE 3.

Assay of the "receptor destroying enzyme" as pathotopic adjuvant.

Vaccination	Antibody against MEL		Infectivity test			Protection (*P*)
	Serum	Bronchi	ΣL	d_x	log *P*	
Ap	340	2·1	24·01	8·77	3·47	2,950
ApRp	362	2·6	23·62	9·16	3·63	4,270
ApRn	287	3·1	21·83	10·95	4·33	21,400
Rn	—	—	34·21	— 1·43	— 0·57	— 4
0 (nil)	—	—	82·78	$d_{10} =$ 2·525	0·00	1

— = < 5 in serum and < 0·5 in bronchi.
A = influenza A vaccine (MEL-strain, formolized; titre: 1,400).
R = "receptor destroying enzyme" of *V. cholerae* (titre: 1,800).
p = peritoneally.
n = nasally.

The results obtained with the "receptor destroying enzyme" (Table 3) are not unequivocal. It is quite clear that RDE, eleven days after its administration, has no effect either on normal antibody production or on the susceptibility of mice to influenza infection (no significant difference between groups *Ap* and *ApRp* on one hand, and between *0* and *Rn* on the other). The main experimental group *ApRn*, however, shows protection values which are far enough from *Ap* not to be classified as identical, yet fail to reach a level which could be considered as significantly different. This finding appears to be reproducible:

on repetition the difference between protection values of groups *Ap* and *ApRn* was $\log_{10}$ 0·97, i.e. ninefold. For purposes of the present study it will suffice to regard RDE as having no satisfactory potentiating effect. Its behaviour, however, points to the existence of substances occupying positions intermediate between full and no potentiating activity. If correct, this would imply that the "nasal" and "extra-respiratory" types of antibody distribution (5 and 0·4 respectively) are not the only possible ones, as tacitly assumed in earlier papers of this series, but the only two hitherto encountered. Since the above findings suggest this possibility but do not provide in any way a conclusive answer, further experimentation in this direction seemed to be warranted. The results will be given in Paper IX.

The *metaperiodate-ion* was given in doses of 0·05 ml. of a 0·01 molal solution, a concentration well tolerated by the animals. This amount given intranasally to live mice is known to leave unaltered the adsorptive capacity of the respiratory tract for the virus. Cellular receptors, however, are *modified* by the action of the chemical—the adsorbed virus particles are incapable of spontaneous enzymatic elution, and the virus-cell combination is rendered a practically irreversible one (Fazekas de St.Groth, 1949). The modifying effect is almost instantaneous (practically completed within three minutes), and in sharp contrast to destruction of receptors by RDE where regeneration is well under way by the end of 24 hours, receptors persist in their modified state for a considerable period of time. As tested in control animals during the present experiment, reparation of the IO_4-effect becomes noticeable only on the sixth day after treatment, and even on the eleventh day, the time of challenging, the *status quo ante* has not yet been attained. The regression curve is not of the expected sigmoid type.

TABLE 4.

Assay of the metaperiodate-ion as pathotopic adjuvant.

Vaccination	Antibody against MEL		Infectivity test			Protection (P)
	Serum	Bronchi	ΣL	d_x	log P	
Ap	297	2·4	27·63	8·09	3·62	4,170
ApMp	308	2·3	27·14	8·58	3·85	7,090
ApMn	243	9·4	22·99	12·73	5·71	513,000
Mn	—	—	35·61	0·11	0·05	1
0 (nil)	—	—	35·72	$d_{10} =$ 2·235	0·00	1

— = < 5 in serum and < 0·5 in bronchi.
A = influenza A vaccine (MEL-strain, formolized; titre: 1,330).
M = potassium metaperiodate (KIO_4, 0·01 M in normal saline).
p = peritoneally.
n = nasally.

The results presented in Table 4 establish the metaperiodate-ion as an effective pathotopic adjuvant. Its nasal administration to mice vaccinated peritoneally against influenza causes a definite shift in the antibody distribution

and results in a hundredfold increase in specific resistance. This is as high as found with any potential adjuvant. The control groups show that the administration of periodate does not interfere either with antibody production or with the infectibility of mice. The latter is in complete agreement with a set of earlier observations (Fazekas de St.Groth and Graham, 1949), according to which there is no difference in the infectivity end-points of different influenza virus preparations whether tested in normal or IO_4-treated eggs or mice.

Assay of compounds altering tissue and capillary permeability.

Histamine, the first substance to be tested in this group, was chosen because it is known to cause pulmonary oedema in most mammalian species. It was realized, however, that the mouse is one of the most unsuitable animals for the study of this effect, since it is remarkably insensitive to the action of histamine under normal circumstances. Thus, though the present experiments were done on normal mice with little hope for positive results, further tests were performed making use of the exaltation of the histamine-effect by the method of Parfentjev and Goodline (1948), and of Halpern and Roux (1949), and these will be reported in the next paper of this series.

TABLE 5.

Assay of histamine as pathotopic adjuvant.

Vaccination	Antibody against MEL		Infectivity test			Protection (P)
	Serum	Bronchi	ΣL	d_x	log P	
Ap	297	2·4	27·63	8·09	3·62	4,170
ApHp	305	2·3	26·69	9·03	4·04	10,900
ApHn	281	2·3	28·42	7·30	3·27	1,860
Hn	—	—	37·01	— 1·29	— 0·58	— 4
0 (nil)	—	—	35·72	d_{10} = 2·235	0·00	1

— = < 5 in serum and < 0·5 in bronchi.
A = influenza A vaccine (MEL-strain, formolized; titre: 1,330).
H = histamine dihydrophosphate (0·02 mg./ml.).
p = peritoneally.
n = nasally.

Histamine had no effect on the immunological behaviour of mice. As is evident from Table 5, groups *Ap*, *ApHp*, and *ApHn* show reactions which differ by less than the variance of the method. The same holds also for the other set of groups, *Hn* and *0*.

Dicoumarin, the second reagent with known action on tissue and capillary permeability, was chosen because of its anti-vitamin K activity. Again, as in the case of histamine, we expected little from the experiment, because owing to the self-imposed restriction of using one standard technique throughout, we intended to give all virtual adjuvants by the nasal route, and in a single dose 11 days before challenging the animals with live influenza virus.

TABLE 6.

Assay of dicoumarin as pathotopic adjuvant.

Vaccination	Antibody against MEL		Infectivity test			Protection (P)
	Serum	Bronchi	ΣL	d_x	log P	
Ap	293	2·7	28·80	10·02	3·96	9,120
ApKp	384	3·1	27·49	11·33	4·44	27,600
ApKn	317	2·9	27·86	10·96	4·31	20,400
Kn	—	—	39·27	— 0·45	— 0·18	— 2
0 (nil)	—	—	38·82	d_{10} = 2·54	0·00	1

— = < 5 in serum and < 0·5 in bronchi.
A = influenza A vaccine (MEL-strain, formolized; titre: 1,400).
K = dicoumarin suspended in normal saline (20 mg./ml.).
p = peritoneally.
n = nasally.

Administered as a crude suspension by the nasal route eleven days before challenging, a 1 mg. dose of dicoumarin does not enhance the protective effect of active immunity, or alter the susceptibility of normal mice to influenza. Twice this amount injected intra-abdominally at the time of vaccination has no effect either on the production of antibody, or its distribution.

It should be stressed that the negative results with this group of substances pertain only to the experimental conditions specified above. Clearly, further tests are required in which dosage, route of administration and timing are so chosen that a pharmacological effect on the day of experimental infection be assured. Until this has been done, evidence regarding the efficacy of histamine and dicoumarin as adjuvants cannot but be considered incomplete and inconclusive.

Assay of astringents as pathotopic adjuvants.

Solutions of three substances known to act as irritants and to induce inflammatory reactions of slightly different type were tested; in addition the suspending medium of influenza vaccines, normal allantoic fluid, was included in this group.

Tannic acid was given in form of a 0·12 p.c. solution in normal saline. This concentration was chosen as the most suitable after a series of sighting experiments. Since these preliminary tests proved rather valuable for the elucidation of the mechanism of pathotopic potentiation, they will be dealt with in detail in the accompanying paper. An intranasal inoculum of 0·05 ml. was well tolerated by our mice, though occasionally on autopsy small areas of consolidation were seen in their lungs, giving a 0·1–0·3 score as a rule (2–8 p.c. of whole surface consolidated), never exceeding 0·8. By the eighteenth day after tannin-treatment these lesions were greyish, transparent, well demarcated, and sunk slightly below the level of the surrounding normal lung tissue; they did not interfere with the evaluation of lesions due to experimental infection with dilutions of influenza virus seven days ago, on day 11.

TABLE 7.

Assay of tannin as pathotopic adjuvant.

Vaccination	Antibody against MEL		Infectivity test			Protection (P)
	Serum	Bronchi	ΣL	d_x	log P	
Ap	340	2·1	24·01	8·77	3·47	2,950
ApTp	305	2·4	24·68	8·10	3·20	1,580
ApTn	247	5·5	20·18	12·70	5·03	101,000
Tn	—	—	31·94	0·84	−0·33	−2
0 (nil)	—	—	32·78	d_{10} = 2·525	0·00	1

— = < 5 in serum and < 0·5 in bronchi.
A = influenza A vaccine (MEL-strain, formolized; titre: 1,400).
T = tannic acid (0·12 p.c. in normal saline).
p = peritoneally.
n = nasally.

As shown in Table 7, tannic acid in the concentration used meets all requirements set for a pathotopic adjuvant. It did not alter the susceptibility of normal mice to influenza, neither had it any appreciable effect on the amount of antibody produced in vaccinated animals. However, when given nasally to immunized mice, it caused a conspicuous shift in the distribution of antibody, and was followed by a highly significant rise in specific resistance.

Zinc sulphate, the next substance to be tested, was given as a 0·10 p.c. solution, again 0·05 ml. volumes intranasally. For purposes of the present study it will suffice to state that this was found to be the optimal concentration; experimental data justifying our choice are presented in Paper IX. This dose of $ZnSO_4$ had no untoward effects on mice; at least they ate normally during the fortnight following treatment, and no macroscopic lesions were seen in the lungs on autopsy either 5, 11 or 18 days after inoculation.

TABLE 8.

Assay of zinc sulphate as pathotopic adjuvant.

Vaccination	Antibody against MEL		Infectivity test			Protection (P)
	Serum	Bronchi	ΣL	d_x	log P	
Ap	293	2·7	28·80	10·02	3·96	9,120
ApZp	243	2·9	27·47	11·35	4·47	26,200
ApZn	327	7·1	24·76	14·06	5·53	338,000
Zn	—	—	36·14	2·68	1·05	11
0 (nil)	—	—	38·82	d_{10} = 2·54	0·00	1

— = < 5 in serum and < 0·5 in bronchi.
A = influenza A vaccine (MEL-strain, formolized; titre: 1,400).
Z = zinc sulphate ($ZnSO_4$, 0·10 p.c. in normal saline).
p = peritoneally.
n = nasally.

In the amount used zinc sulphate acted as pathotopic adjuvant. The findings are very similar to those obtained with tannin solutions: the increase in bronchial antibody titres and protection-values is unequivocal, and the agreement between groups *Ap* and *ApZp* is satisfactory. However, there is one blemish in Table 8: the protection-value of group *Zn* is 11 times as high as that of the normal control group (*0*). When assessing the significance of this finding, it should be kept in mind that the challenging virus preparation was of exceptionally high infectivity (total lung lesions 38·82, as opposed to a usual 32 to 36), and gave 100 p.c. scores in several of the groups of non-immunized animals infected with large doses of virus, i.e. killed all mice within five days. Since values on the flat ends of the sigmoid dose-response curve weigh heavily on the average, the occurrence of such readings introduces the same type of inaccuracy as the presence of groups with no macroscopic lesions. The latter possibility was considered in the original description of the protection test (Fazekas de St.Groth and Donnelley, 1950c), and it was concluded that in these instances the significance of differences is inversely related to the number of groups with zero readings. Obviously, the same holds also for groups with 100 p.c. readings, and hence the big difference found between groups *Zn* and *0* is regarded as partly due to chance. This decision is the easier to make since in two replicate experiments protection-values for group *Zn* were 6 and 7, respectively.

Soluble starch was the third irritant tested, given as a 0·5 p.c. suspension in normal saline. The animals tolerated this treatment; neither clinical signs of distress were observed, nor gross pulmonary lesions on autopsy. Choice of the dose was based on exploratory experiments: larger doses killed mice within 48 hours when given intranasally, smaller ones did not seem to have any advantage as pathotopic adjuvants.

TABLE 9.

Assay of soluble starch as pathotopic adjuvant.

Vaccination	Antibody against MEL		Infectivity test			Protection (*P*)
	Serum	Bronchi	ΣL	d_x	log *P*	
Ap	293	2·7	28·80	10·02	3·96	9,120
ApSp	361	2·9	29·41	9·41	3·72	5,250
ApSn	245	2·7	29·11	9·71	3·83	6,760
Sn	—	—	37·98	0·84	0·33	2
0 (nil)	—	—	38·02	$d_{10} =$ 2·54	0·00	1

— = < 5 in serum and < 0·5 in bronchi.
A = influenza A vaccine (MEL-strain, formolized; titre: 1,400).
S = starch solution (0·5 p.c. in normal saline).
p = peritoneally.
n = nasally.

The findings presented in Table 9 show that the starch suspension did not potentiate immunity, indeed it had no effect on any of the reactions tested in the standard experiments.

Normal allantoic fluid has already been used in previous experiments (Fazekas de St.Groth and Donnelley, 1950), then as a control for vaccines, i.e. after treatment with 0·05 p.c. formaldehyde and an incubation at 37° C. for 12 hours. In the tests recorded in Table 10, the preparation used was a pool of allantoic fluids from 13 days old White Leghorn embryos. The only pre-treatment consisted in light spinning to remove contaminating erythrocytes and cellular debris.

TABLE 10.

Assay of normal allantoic fluid as pathotopic adjuvant.

Vaccination	Antibody against MEL		Infectivity test			Protection (P)
	Serum	Bronchi	ΣL	d_x	log P	
Ap	316	2·9	25·88	8·41	3·84	6,920
ApFp	241	2·7	27·14	7·15	3·27	1,860
ApFn	290	2·7	26·73	7·56	3·46	2,880
Fn	—	—	34·77	— 0·48	— 0·22	— 2
0 (nil)	—	—	34·29	d_{10} = 2·19	0·00	1

— = < 5 in serum and < 0·5 in bronchi.
A = influenza A vaccine (MEL-strain, formolized; titre: 1,600).
F = normal allantoic fluid.
p = peritoneally.
n = nasally.

The results were negative: normal allantoic fluid does not alter the susceptibility of immune mice to homologous challenge, neither does it interfere with the infectivity of influenza virus in normal mice.

DISCUSSION.

Resistance to influenza virus infection appears to be determined by the amount of specific antibody free in the respiratory tract (Fazekas de St.Groth and Donnelley, 1950b, c). Accordingly, increased protection can be hoped for either from enhanced antibody production, or from a relative increase in antibody concentration at the immediate environment of infectible cells. The second possibility was realized by what we termed *pathotopic potentiation* of immunity (Fazekas de St.Groth and Donnelley, 1950d; Fazekas de St. Groth, 1950b), the nasal administration of an unrelated influenza preparation to animals vaccinated by extrarespiratory routes. This treatment induced a shift in the normal distribution of antibody, resulting in higher concentrations in the respiratory tract, and in a considerably elevated protection.

In the search for *pathotopic adjuvants*, i.e. agents capable of causing a local accumulation of antibody when applied at the site of possible infection, a number of compounds was tested. All of these, though mostly unrelated to each other, were chosen because of their similarity in at least one aspect to the virus of influenza, the first pathotopic adjuvant. The potentiating effect was assessed by two criteria. First, the efficacy in shifting the antibody distribution in favour of the bronchi; this was done by the comparison of serum and bronchial antibody titres before and after nasal instillation of the substance to be tested. Secondly, whether or not specific resistance of peritoneally vaccinated mice was enhanced by the "adjuvant"; this was determined by challenging with graded doses of live virus.

Obviously, the two criteria are distinct only insofar as different methods of assay are involved. If resistance is a direct function of the amount of antibody present at the site of infection, it can be expected that the two methods give identical results. The actual experimental findings indicate that such is the case. All agents which proved to be active as pathotopic adjuvants were found so in both types of assay, while compounds ineffective gave negative results by either test. This complete parallelism constitutes a further confirmation of our original thesis that only that fraction of antibody is *immunologically active* (i.e. capable of preventing infection) which meets and neutralizes the virus before it could enter a susceptible cell (Paper IV).

In order to eliminate complicating variables, formal proof is required that (1) the substances tested did not interfere with the production of antibodies, and that (2) their administration did not alter the susceptibility of the experimental animals to the challenging strain of virus.

It is evident from the serological results that none of the compounds tested had any effect on the amount of antibody produced. There is no significant difference between serum titres of the different experimental groups receiving the same influenza vaccines. The changes—if present—are restricted to the distribution of antibody. This finding is in full agreement with those reported previously (Papers V and VI), and shows that the main feature of pathotopic potentiation is the local accumulation of antibody at the level of infectible cells.

In the series of control experiments where the "adjuvant" was given to non-immunized mice, the animals so treated proved to be as susceptible to subsequent infection with the MEL strain of influenza A as the group of normal control mice. This finding is not surprising in the case of RDE, since it has been demonstrated that enzymically-destroyed receptors would regenerate within six days (Fazekas de St.Groth, 1948a), and after this time the animals were again fully susceptible to infection (Stone, 1947; 1948a; 1948b). Neither does the modification of cellular receptors by the metaperiodate-ion alter the host's susceptibility (Fazekas de St.Groth, 1948b; Fazekas de St.Groth and Graham,

1949). Scalfi and Olia (1949) have shown that intranasal instillation of concentrated solutions of tannin does not prevent either the fixation of the virus to cells, or the infection of the respiratory tract. Data concerning the other substances tested are not available in the literature, but in our experiments their influence on the infectibility of the host eleven days after administration was not detectable. It is interesting to note that this holds also in the case of live vaccinia virus which is capable of multiplying in the lungs, and could be re-isolated in most instances at the time of challenging with influenza virus. This cannot but mean that the two viruses do not interfere with each other's growth, either because they parasitize different cells, or if not then at least that their reproduction is supported at the expense of different metabolic systems of the same cell. Such simultaneous parasitism has been demonstrated for several pairs of unrelated viruses.

Agents enhancing specific resistance were encountered in three of the four experimental groups, while all groups contained substances which failed to show significant activity. As far as it provides a range of potential adjuvants, this finding is promising from the practical point of view. However, the mechanism underlying the pathotopic potentiation of immunity appears rather obscured than clarified by the results. It would be pointless, therefore, to discuss the mode of action of these substances at this stage without a more exacting scrutiny of their behaviour in the animal organism. Diverse experiments designed specially to elucidate these problems form the substance of the next paper in this series. The search for a common denominator for all adjuvants and a fuller treatment of the theoretical aspects of pathotopic potentiation is deferred till then.

SUMMARY.

Four groups of agents, each resembling one aspect of the influenza virus, were tested to see whether they were capable of enhancing specific resistance when given by the nasal route to immune mice.

Of the viruses vaccinia was effective; bacteriophage C-16 was not.

Of agents acting on cellular virus receptors the metaperiodate-ion was highly active, while the "receptor destroying enzyme" of *V. cholerae* gave equivocal reactions.

The two agents supposedly increasing capillary and tissue permeability did not produce the expected pharmacological effect under conditions of the test; they were also inactive as pathotopic adjuvants.

Appropriate concentrations of certain astringents (zinc sulphate, tannin) brought about pathotopic potentiation of immunity, while another (soluble starch) did not; normal allantoic fluid was similarly ineffective.

All adjuvants which enhanced immunity also proportionately increased the amount of specific antibody in the respiratory tract.

None of the reagents altered the susceptibility to influenza of non-immunized mice, and the ones effective were so only when given by the nasal route.

REFERENCES.

Burnet, F. M. and Stone, J. D. (1947): Austral. J. exp. Biol., 25, p. 227.
Fazekas de St.Groth, S. (1948a): *Ibid.*, 26, p. 271.
Fazekas de St.Groth, S. (1948b): Nature, 162, p. 294.
Fazekas de St.Groth, S. (1949): Austral. J. exp. Biol., 27, p. 65.
Fazekas de St.Groth, S. (1950a): *Ibid.*, 28, p. 15.
Fazekas de St.Groth, S. (1950b): *Ibid.*, 28, p. 559.
Fazekas de St.Groth, S. (1951): *Ibid.*, 29, p. 339.
Fazekas de St.Groth, S. and Donnelley, M. (1950a): *Ibid.*, 28, p. 31.
Fazekas de St.Groth, S. and Donneley, M. (1950b): *Ibid.*, 28, p. 45.
Fazekas de St.Groth, S. and Donnelley, M. (1950c): *Ibid.*, 28, p. 61.
Fazekas de St.Groth, S. and Donnelley, M. (1950d): *Ibid.*, 28, p. 77.
Fazekas de St.Groth, S. and Graham, D. M. (1949): *Ibid.*, 27, p. 83.
Fenner, F. (1947): *Ibid.*, 25, p. 257.
Halpern, B. N. and Roux, J. (1949): C. r. Soc. Biol., 143, p. 923.
Parfentjev, I. A. and Goodline, M. A. (1948): J. Pharm. exp. Ther., 92, p. 411.
Scalfi, L. and Olia, A. (1949): Studi Sassaresi, 27.
Stone, J. D. (1946): Austral. J. exp. Biol., 24, p. 197.
Stone, J. D. (1947): Nature, 159, p. 780.
Stone, J. D. (1948a): Austral. J. exp. Biol., 26, p. 49.
Stone, J. D. (1948b): *Ibid.*, 26, p. 287.

STUDIES IN EXPERIMENTAL IMMUNOLOGY OF INFLUENZA

IX. THE MODE OF ACTION OF PATHOTOPIC ADJUVANTS

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The protective efficacy of extra-respiratory vaccination with influenza virus preparations can be enhanced markedly by what we termed *pathotopic potentiation* of immunity (Fazekas de St.Groth and Donnelley, 1950c). This method involves the administration to immunized animals of an "adjuvant" at the site of possible infection, viz. the respiratory tract in the case of influenza virus. The nasal instillation of such *pathotopic adjuvants* brings about a shift in the normal distribution of antibody, resulting in a higher concentration in the immediate environment of infectible cells. It could be shown that several, seemingly unrelated substances proved to be active while others, for no obvious reason, were ineffective (Fazekas de St.Groth, Donnelley and Graham, 1951).

It is the aim of this study to inquire further into the mechanisms underlying the potentiating effect. The work progressed in parallel with the experiments of the preceding paper; it did not follow any predetermined course—it is essentially a collection of complementary experiments, excursions and deviations, such as became necessary as the main work yielded results unexpected or incompatible with some of the preconceived ideas which were part of our working hypothesis. Since each step in experimentation was conditioned by and is a logical outcome of the previous step, it would be futile to maintain the usual pretence of being unbiased by one's findings, and present observational and inferential findings under separate headings, viz. "Experiments" and "Discussion". The description will follow the chronological order, and thus be of the same pattern as the experimental part of the preceding paper.

All *materials and methods* have been described previously in this series; references, and if necessary, additional details will be given when dealing with the particular experiments.

Behaviour of the virus of vaccinia and of Shigella-phage C-16 in the mouse.

The first and most incomprehensible finding of the previous paper concerned the complete failure of the bacterial virus to act as pathotopic adjuvant. This was in sharp contrast with the marked activity shown by both the virus

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of vaccinia and different strains of influenza virus. The latter were tested in the active and in the heat- or formol-inactivated state and were found equally effective in either form.

A reason for the observed discrepancy might have been the instability of the phage preparation or its neutralization by some component of the animal organism. In the first experiment, therefore, the viability of the phage was tested (a) after exposure to 37° C. for 24 hours, and (b) after mixing and incubating for 30 minutes with the serum or ground lung tissue of mice. Table 1 gives the results; parallel experiments with the viruses of influenza and vaccinia are included for the sake of comparison.

TABLE 1.

Effect of heat and tissue extracts on viral infectivity.

Virus	Treatment	Infectivity titre (log_{10})	Reduction factor*
Vaccinia (Gillard strain)	heat	4·30	1/40
	serum	5·30	1/4
	lung tissue	6·10	1·6
	nil (control)	5·90	1
Bacteriophage (C-16 strain)	heat	10·30	1
	serum	10·60	1·6
	lung tissue	10·30	1
	nil (control)	10·40	1
Influenza (MEL strain)	heat	5·50	1/710
	serum	7·50	1/7
	lung tissue	7·67	1/5
	nil (control)	8·35	1

* Fivefold changes are significant.

Treatments: *"heat"* = 1:10 dilution of virus in saline incubated at 37° C. for 24 hours.
"serum" = 1:10 dilution of virus in normal mouse serum incubated at 37° C. for 30 minutes.
"lung tissue" = 1:10 dilution of virus in homogenate of normal mouse lungs incubated at 37° C. for 30 minutes.
"control" = average of two titrations, one performed before the experiment, the second in parallel with the other tests.

It is evident that the phage is quite stable at 37° C., and is not neutralized at all by either the serum or the ground lung tissue of mice. Indeed, its infective capacity is less affected by these treatments than that of the virus of vaccinia, or especially, the strain of influenza virus used in the experiment.

The fact that infectivity is not neutralized does not necessarily exclude some interaction between tissue components and the virus. It is well known, in the case of many species of animal viruses, that "inhibitors" or substances causing "non-specific reactions" can imitate antibodies when tested by *in vitro* methods; most of these, however, do not exhibit neutralizing properties or only to a disproportionately lower degree when assayed in infectivity titrations.

In consequence, it seemed worth while to investigate the behaviour of the bacterial virus in the mouse by some other, independent method. The test for antibody production seemed suitable, since it is known, for example, that in rabbits both the phage C-16 and the virus of

vaccinia are good antigens and induce the production of specific antibodies. In the actual experiments the two viruses were given by both the peritoneal and nasal route (as has been done in the work described in the preceding paper). Results of the antibody titrations performed on sera eleven days after inoculation can be seen in Table 2.

TABLE 2.

Serum antibody titres after different routes of immunization.

Antigen	Route	Dose*	Antibody titre§
Vaccinia virus	nasal	10^3	238
	peritoneal	$10^{5.6}$	61
Bacteriophage	nasal	10^{10}	$< 0{\cdot}5$
	peritoneal	$10^{10.3}$	137

* Expressed as infective doses for the respective host.

§ Vaccinia antibody titrations were performed *in vitro*, against 4 haemagglutinating doses; phage antibody was titrated by the neutralization-test, against 20 infective doses.

Here we have an unexpected finding. The bacteriophage served as an excellent antigen when injected peritoneally, but failed to elicit any appreciable antibody response when instilled intranasally. The virus of vaccinia proved to be equally effective by either route.

One possible interpretation, amenable to immediate experimental testing, is to assume that there are no receptors for the bacteriophage in the respiratory tract of the mouse. Were this the case, the phage would be incapable of combining with the cells lining the respiratory tract. Obviously, the failure of virus-cell combination would also prevent the transgression by the virus of a barrier consisting of the selfsame cells.

TABLE 3.

Adsorption of viruses in excised lungs.

Virus	Infectivity titre*		Reduction factor§
	Before adsorption	After adsorption	
Vaccinia (Gillard strain)	7·0	6·5	1/3
	5·0	4·0	1/10
	3·0	1·5	1/32
Bacteriophage (C-16 strain)	11·5	11·8	2
	7·5	7·0	1/3
	3·5	3·8	2
Influenza (MEL strain)	9·5	6·6	1/790
	6·5	4·0	1/320
	3·5	0·4	1/1,200

* Infectivity expressed in $\log_{10}$ units per 0·50 ml.

§ Fivefold changes are significant.

The crucial experiment was performed on excised lungs by the technique previously described (Fazekas de St.Groth, 1948a). Various quantities of the viruses were aspirated through the tracheal cannula and left in contact with the respiratory tract for 30 minutes. At the end of this period the fluid was expelled, and the respiratory tract washed with three changes of 0·5 ml. saline. The amount of virus present in the pooled fluids was titrated by infectivity tests on the respective host cells.

It can be seen from Table 3 that the virus of vaccinia is capable of combining with the lining of the respiratory tract of mice. The adsorption of the virus is, however, considerably less complete than of influenza viruses, of which 10^9 infective particles are adsorbed without difficulty. The bacterial virus, on the other hand, shows no adsorption whatsoever. Even the smallest number of phage particles failed to be bound by the lining of the bronchial tree; the recovery from the washing fluids was complete.

On the grounds of the above experiments it can be stated that Shigellaphage C-16 had sufficient reason not be active as a pathotopic adjuvant in the mouse, since it never really entered the organism after having been instilled intranasally. Casual contacts with the mouse's tissues resulting from Brownian movement are obviously insufficient to render the phage effective, either by the criterion of antibody formation or by that of pathotopic potentiation. The results are satisfactory insofar as they provide a reasonable explanation for a discrepancy; however, they give no clue to the mode of action of the effective viruses. The conclusion so far is not more than could have been deduced from first principles: no agent can be active unless present at the site of its supposed action.

Differences in the action of the "receptor destroying enzyme" of V. cholerae *and the metaperiodate-ion on the lungs of living mice.*

The second group of substances had its specific action on cellular receptors as common feature. Of the two the periodate-ion caused pathotopic potentiation of immunity to a highly significant degree; reactions following the nasal administration of RDE were equivocal, and the question whether it acted as a pathotopic adjuvant was left unanswered.

The two reagents have different modes of action, viz., oxidation of presumably α-glycols by IO_4 with the production of aldehyde groups, and enzymic action of RDE without the production of a comparable amount of reducing substances. From a biological viewpoint, the action of RDE is more drastic since it destroys receptors (renders the surface incapable of adsorbing virus particles), while the action of periodate—in the concentrations used—only modifies them, i.e. does not interfere with the adsorption of the virus but prevents its spontaneous elution. Only considerably larger quantities of periodate have a destructive effect. Since on the grounds of this consideration the reverse of the actual experimental findings should be expected, one is forced to conclude that the potentiating effect is not merely another aspect of the specific action of the two agents on cellular receptors, but has to be considered as an unrelated effect on the respiratory tract of the mouse.

From earlier work it was known that the regeneration of receptors follows different patterns depending on whether they have been destroyed by RDE or modified by IO_4. After

RDE-treatment regeneration starts within 12 hours and is well advanced after 24; the normal level is reached within six days (Fazekas de St.Groth, 1948b). After periodate-action there is no appreciable regenerative change in the state of receptors for at least 96 hours, and full restitution takes at least a fortnight (Fazekas de St.Groth and Graham, 1949). Hyper-regeneration, the rule after RDE-treatment, was not observed after periodate. Consequently, if the potentiating effect were related to the host's responsive reactions, RDE might be active but only for a shorter period than periodate. This possibility was tested in the following experiment.

Mice were immunized peritoneally with killed MEL (A) virus vaccine, and given intranasal inocula of 0·05 ml. RDE (titre 2,000) or KIO_4 (0·01 M) 28, 21, 14, 7, 4, 2 and 1 days before challenging with live MEL virus. The protection-test was conducted and evaluated as in previous papers of this series (original description Fazekas de St.Groth and Donnelley, 1950b).

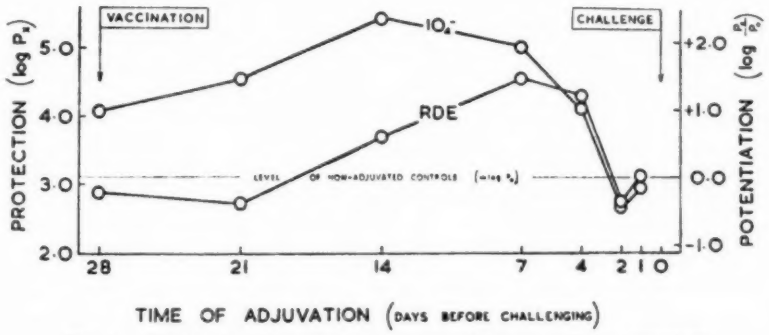

Fig. 1. Pathotopic potentiation by agents acting on cellular receptors (for full description see text).

Fig. 1 shows that the difference between the potentiating action of the metaperiodate-ion and RDE is quantitative rather than qualitative. Both serve as pathotopic adjuvants for a certain period of time, periodate however being superior both in regard to the time span of potentiating activity and the degree to which it was capable of shifting antibody distribution.

The implications of this finding are rather far-reaching. It follows that potentiation is not an all-or-nothing type of reaction, and all gradations between full and no response are possible. Further, that since the two compounds tested are known to have only a limited time of action while their potentiating effect becomes manifest after a certain lag and is of long duration, it should be characteristic not so much of the particular treating agent but of the response of the tissues affected. Accordingly, it would seem less promising to look for the explanation among the material properties of pathotopic adjuvants, as has been done in the absence of any better lead in the preceding paper. A more reasonable approach would be to expect a common type of response on the side of the organism, some reaction which could be brought about by widely different methods.

The part of capillary and tissue permeability in the potentiating effect.

In the systematic screening of potential pathotopic adjuvants (cf. preceding paper) both histamine and dicoumarol were found to be without effect. Methodical as those tests might have been, they could hardly be regarded as adequate, since no provision was made to ensure full pharmacological action of the agents under the experimental conditions. Indeed, it is known that, on the one hand, normal mice are singularly insensitive to histamine and that, on the other, dicoumarol has to be administered by the enteral route for maximum efficacy. These shortcomings were corrected in the following tests.

From the work of Parfentjev and associates (Parfentjev and Goodline, 1948; Parfentjev and Schleyer, 1949; Parfentjev, 1950) and of Halpern and Roux (1949; 1950) it is known that mice can be rendered highly sensitive to histamine by pretreatment (immunization) with large doses of *H. pertussis* phase I vaccines. The essential findings of these authors were readily confirmed, and their method was adopted for further tests with histamine.

Three partly overlapping experiments were done. In the first the mice vaccinated against influenza A received a sensitizing dose, 60×10^9 organisms of formolized *H. pertussis* phase I suspended in 1·0 ml. peritoneally[2]. After five days 0·1 mg. of histamine (dihydrophosphate salt) was instilled nasally, and two days later all groups were challenged with dilutions of active influenza A virus.

Rather unexpectedly, not only the animals sensitized to and receiving histamine showed some enhancement of immunity, but also the control group given pertussis vaccine only. The second experiment, in which histamine was given by the peritoneal route and more extensive controls were included, yielded similar results (Table 4).

TABLE 4.

Effect of histamine on resistance to influenza.

Treatment	Infectivity test			Protection (*P*)
	ΣL	d_x	log *P*	
A^{14}	24·47	9·79	3·15	1,410
A^7 P^5 H^2	20·75	13·51	4·92	83,200
A^7 P^7	20·17	14·09	5·12	132,000
P^5 H^2	37·64	— 3·38	— 1·19	— 15
P^7	36·92	— 2·66	— 0·97	— 9
H^2	34·86	— 0·60	— 0·22	— 2
nil (control)	34·26	d_{10} = 2·755	0·00	1

A = influenza A vaccine (titre 1,400); 0·10 ml. peritoneally.
P = *H. pertussis* (phase I) vaccine; 60×10^9 organisms peritoneally.
H = histamine dihydrophosphate; 0·1 mg. peritoneally.
The *index numbers* show the interval, in days, between consecutive treatments.

It is evident that mere vaccination with killed *H. pertussis* preparations followed by 0·25 LD_{50} of histamine (group P^5H^2) does not protect mice against influenza. The same treatment, if applied to animals actively immune to influenza, will produce a highly significant rise in specific resistance.

[2] Pertussis vaccines were kindly prepared and made available through the courtesy of Mr. M. F. Warburton, of the Commonwealth Serum Laboratories.

This potentiation of immunity, however, cannot be considered *pathotopic* in the strict sense of the term, as the local (pathotopic) administration of the adjuvant was not essential; enhancement of immunity was brought about as readily by the peritoneal injection of histamine as—in the previous experiment—by its nasal instillation. Indeed, the effect cannot even be ascribed to the dose of histamine given experimentally; as group A^7P^7 shows, sensitization with pertussis vaccine by itself was just as effective. In this instance the production of histamine has been effected presumably by the experimental animal itself. The reason for the assumption that histamine is necessary at all lies in the fact that in a third experiment the potentiating effect in groups $A^7P^5H^2$ and A^7P^7 could be considerably reduced, though not completely prevented, by the daily administration of antihistaminics. This latter observation is in qualitative agreement with the findings of Parfentjev and the French authors, though the effective dose in the present case appeared to be considerably higher than that required for the prevention of lung edema and death[3]. The fact that *only* phase I vaccines were capable of sensitizing to histamine, but not vaccines prepared from avirulent strains, has been confirmed.

It is worth mentioning, as it gives a valuable clue to the mechanism involved, that in all groups which received phase I *H pertussis* vaccine, irrespective of whether they were infected with influenza or not, at least 60 p.c. of the animals showed edematous lungs on autopsy, and an accumulation of serofibrinous transudate in their pleural and peritoneal cavities. Such transudates were absent after histamine alone or after the injection of phase IV pertussis vaccines. It does not seem too far-fetched an inference that the potentiating effect is related to this phenomenon inasmuch as it rests on an increased capillary and/or tissue permeability. Accordingly, one could envisage an oozing out of plasma into the intercellular space, which in the case of pulmonary alveoli would mean direct entry into the air space, as recent concepts on alveolar structure converge on the view that at this level the barrier between the circulation and the lumen consists of a single layer of capillary endothelium with additional amorphous plaques and occasional sedentary mesenchymal cells. In immune animals, of course, such a mechanism could well account for the increased movement of antibody from the circulation into the respiratory tract, thereby enhancing resistance to pneumotropic pathogens.

Experiments with dicoumarin point to the same mechanism. In these tests, unlike those in the preceding paper, the route of administration used was not the nasal but the one most effective from the pharmacological point-of-view, viz. the enteral. Dicoumarin was added to the food of the animals. The actual intake has not been determined with certainty; had the

[3] In a recent report Biozzi, Mené and Ovary (C. r. Soc. Biol., 144, p. 503.) noted the partial failure of antihistaminics to suppress increased capillary permeability provoked by various means.

animals taken the maximum, it would have amounted to 15 mg./day for a mouse of 23 gm. weight. The treatment was continued for a period of 11 days between vaccination and challenging with influenza A virus. Other groups of animals were given daily peritoneal injections of 5 mg. dicoumarol crudely suspended in saline for the 6 days preceding experimental infection.

TABLE 5.

Effect of dicoumarin on resistance to influenza.

Treatment	Infectivity test			Protection (*P*)
	ΣL	d_x	log *P*	
A	30·04	6·60	2·46	288
A Do	24·38	12·26	4·57	37,200
A Dp	26·16	10·48	3·91	8,140
Do	38·47	— 1·83	— 0·68	— 5
Dp	36·97	— 0·33	— 0·12	1
nil (control)	36·64	d_{10} = 2·68	0·00	1

A = influenza A vaccine (titre 1,240); 0·10 ml. peritoneally.
Do = dicoumarin administered orally for 11 days.
Dp = dicoumarin injected peritoneally for 6 days.

A considerable fraction (approximately 20 p.c.) of the animals receiving dicoumarin in their food died during the experimental period; on autopsy all of these showed extensive bleedings in serous and mucous membranes, less frequently in parenchymatous organs and in the loose connective tissue of the subcutis. Similar findings, though less regularly, were also obtained in the group which was given dicoumarin by the peritoneal route. The extent of extravasation seemed to be roughly correlated with the degree of potentiating activity, as presented in Table 5. Thus, though an indisputable enhancement of active immunity could be witnessed, dicoumarin—just as histamine—cannot be regarded as *pathotopic* adjuvant, and for the same reason. The action of both agents is systemic in character rather than restricted to the site of future infection, and hence does not fulfill the requirements of localized action.

Because of this generalized effect both agents tested in this group, though capable of potentiating specific resistance, are unsuited as adjuvants; the effective dose is very close to the lethal dose, and harmful side effects would more than counterbalance the advantageous shift in the distribution of anti-influenzal antibodies.

Astringents as pathotopic adjuvants.

Experiments under this heading, unlike the rest of the present paper, were done before embarking on the main tests published in the previous

paper. This preliminary experimentation was necessary because we could not find reports in the literature providing adequate information on the dosage and effects of the selected agents when given by the nasal route.

After determining the minimal lethal dose for the three reagents when given by the nasal route, the dilutions to be tested were so chosen that less than 10 p.c. of the animals should be lost due to this treatment. Thus 0·05 ml. nasal inocula of 0·8 p.c. tannin, 0·3 p.c. zinc sulphate and 1·0 p.c. soluble starch were given to peritoneally-immunized mice, and the protection test carried out as usual. The results were disappointing; none of the groups showed enhanced resistance; if anything, the control mice which received no adjuvant scored higher protection values than the adjuvated groups.

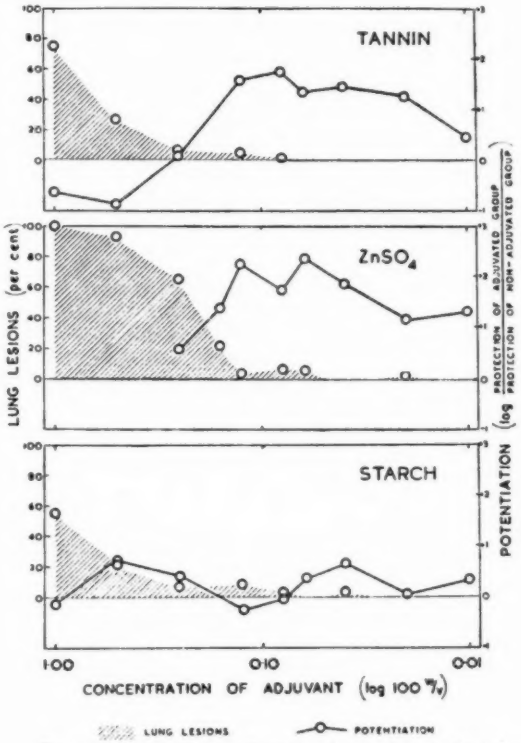

Fig. 2. Potentiation of active immunity against influenza by intranasal instillation of astringents.

The matter would have been allowed to rest there, but for the reason that by now the working hypothesis postulated adjuvating effect from any treatment capable of increasing capillary permeability. Increased capillary permeability is a characteristic of inflammations, and astringents cause inflammation. Obviously there was a false step in the argument, and this called for closer scrutiny. Since inflammation was undoubtedly present (histological picture), the failure might have been due to the unknown temporal or quantitative aspects of the process. Exploratory experiments were devised therefore along these two lines.

In the first set of tests the severity of inflammation was varied, the time between the nasal administration of the astringent and the challenging of the animals being kept constant at ten days.

The results obtained (Fig. 2) gave a satisfactory answer to the question: both the height of inflammation and the potentiating effect depend on the dose

given, but they have different maxima and the regression curves are quite distinct and independent. The effective range for tannin proved to be between 0·15 and 0·02 p.c., for $ZnSO_4$ between 0·20 and 0·01 p.c., while soluble starch failed to show any activity over the whole range tested. In parallel with this experiment the histological changes brought about by the astringents were also determined; this investigation will form part of a later communication concerned with the pathology of influenza in immune animals (Bate and Fazekas de St.Groth, 1951). Pertinent to this point is the observation that whenever intracapillary or intralymphatic clotting of fibrin was present in the lungs, the inflammation did not lead to enhanced resistance. In other words, it is only a *mild* inflammatory process which is accompanied by the local accumulation of antibody and which, under special conditions, manifests itself in pathotopic potentiation of immunity.

Results of the second type of experiment, in which the dose of the reagents was kept constant and the time of administration varied (as has been done earlier with RDE and IO_4; cf. Fig. 1.), did not yield new information relevant to the mechanism under study. These tests fall rather naturally within the scope of a forthcoming paper in which the time span of potentiating activity was investigated by different methods.

Accumulation of foreign material in the lungs after nasal administration of pathotopic adjuvants.

Experiments described in the previous sections dealt with the immediate problems arising in the course of the main work on pathotopic adjuvants (Fazekas de St.Groth, Donnelley and Graham, 1951). Besides resolving some contradictions they also revealed hitherto unknown elements of the mechanism, and thus provided material for the construction of a more adequate hypothesis. The final set of tests aimed at furnishing direct and formal proof for the new interpretation, viz. the demonstration of increased capillary or tissue permeability in the lungs after the administration of some of the effective adjuvants.

At first the classical technique employing a colloidal dye was attempted, but it was found difficult to obtain quantitative results by this means. I had the impression that both trypan blue and Indian ink accumulated to a greater extent in the lungs of mice treated with the adjuvants; however, it was not possible to assess the amount reaching the lumen of the respiratory tract. Histological sections showed rather patchy distribution, and bronchial washings taken from such lungs did not contain coloured material sufficient for macroscopic estimation.

A second type of experiment proved more successful. It has been shown by Henle and Henle (1946) that large doses of active influenza virus injected into the peritoneal cavity of mice caused occasionally pulmonary consolidation, typical of experimental infection by the nasal route. The tentative interpretation of this finding supposed that lungs could be infected also from the vascular side. However, already in earlier work (Fazekas de St.Groth and Donnelley, 1950a) the alternative explanation was suggested that mice have become in-

fected from the bronchial side by the minute fraction of peritoneally injected virus which found its way into the lumen of the respiratory tract. Results of experiments set up purposely to decide between the alternatives seemed to give further support to the latter view.

Isaacs and Gunter (personal communication) found that influenza viruses inoculated intravenously were adsorbed to the vascular wall, and their adsorption could be prevented by previous injection of the "receptor destroying enzyme" (RDE) of *V. cholerae*. As it is known that destruction of cellular receptors equals prophylaxis against influenza infection (Stone 1947, 1948a, b), groups of mice were given 10,000 units of RDE either intravenously or intranasally, and were inoculated six hours later with 1·0 ml. of high titre MEL virus by the peritoneal route. Of the group receiving RDE intravenously 17/30 mice showed pulmonary consolidation on the seventh day, but only 2/30 of the group given RDE nasally. This means that receptor destruction on the bronchial surface was considerably more effective in preventing infection than on the vascular side, and hence the site of infection was presumably at the bronchial surface of the susceptible cells. By accepting this conclusion the principle of a sensitive method is given by which the lung lesions developing after peritoneal injections of influenza virus serve as a measure of the permeability of the barrier between the circulation and the lumen of the respiratory tract.

In the actual experiments vaccinia virus, KIO_4 or $ZnSO_4$ was given in optimal amounts (see above) to groups of 10 mice, and four days later 0·5 ml. of active SW virus titreing 800 by the standard haemagglutination technique (= $10^{9.3}$ infective doses), peritoneally. Lung lesions were evaluated in the usual way after 10 days. Phage C-16 and normal saline were instilled nasally in the control groups.

TABLE 6.

Permeability of lungs after treatment with various pathotopic adjuvants.

Pretreatment		Lung lesions
Agent	Dose	
Vaccinia virus	$10^{4.2}$ particles in 0·05 ml.	74·0 p.c.
Bacteriophage C-16	$10^{9.6}$ particles in 0·05 ml.	6·4 p.c.
Potassium periodate	0·01 M, 0·05 ml.	57·5 p.c.
Zinc sulphate	0·08 p.c., 0·05 ml.	46·0 p.c.
Physiological saline	0·05 ml.	9·1 p.c.
Nil (control)	—	8·5 p.c.

Four days after nasal instillation of the treating agent all mice were given $10^{9.3}$ egg-infective doses of SW influenza virus peritoneally; lung lesions were assessed ten days later.

All groups which received nasal inocula that would have been ineffective as pathotopic adjuvants showed only trivial lung lesions (less than 10 p.c. of the lung area, and in not more than half of the animals), while those treated with effective adjuvants had pulmonary consolidation averaging around 60 p.c., all animals being involved. Such a rise would be equivalent to an approximately fiftyfold increase in virus concentration, were the animals infected by the nasal route.

Too much stress should not be laid, however, on the quantitative aspects—the enormous difference in size between the virus particle and an antibody molecule precludes any direct

comparison. Further, the fact that vaccinia virus is apparently more effective in facilitating the transgression of influenza virus than is the metaperiodate-ion or zinc sulphate, hints at differences also in the qualitative details. Of importance at this point is only the demonstration that the permeability of lungs for particles even as large as an influenza virus is increased by a treatment which, as has been assumed, renders them more permeable to specific antibodies.

CONCLUSIONS.

The diversity of experiments and findings only serves to underscore the few general conclusions that can be drawn, and which hold for the entire group. First, that the enhancement of immunity against experimental influenza infection by pathotopic adjuvants is not an all-or-nothing type of reaction, but exhibits both quantitative and temporal gradation. Some of the agents were completely ineffective (bacteriophage C-16, soluble starch, etc.), others showed ephemeral action (the "receptor destroying enzyme" of *V. cholerae*) or acted only under such special conditions as to render them unsuitable for practical application. Those found effective (influenza vaccines, the virus of vaccinia, the metaperiodate-ion and certain astringents) had definite concentration optima and characteristic time spans of action.

The heterogeneity of the last group suggests forcibly that the common denominator for their action is to be looked for neither among the material properties nor in the specific activity of these unrelated substances, but rather in the response of the animal organism to their presence. For lack of a better word this response will be called *mild inflammation*, realizing though that for all the morphological similarities it might be quite different, even qualitatively, from the classical picture of the pathologist. It is certainly true that severe inflammation following higher doses of tannic acid or $ZnSO_4$, characterized by foci of necrotized cells, extensive mobilization of mesenchymal elements, demarcation of the affected area with intravascular clotting of fibrin, does not lead to the enhancement of immunity by the accumulation of available antibody. Lower concentrations of the same reagents, notwithstanding their failure to induce vehement tissue reactions, serve as good pathotopic adjuvants. The early, exacting work of Fox (1936a, b) on the fixation of foreign materials (among others, antibodies) in lungs after intrabronchial irritation also shows that localization does not depend on the severity of inflammation. Silber and Baidakova (1945) furnished evidence that influenza virus preparations were capable of rendering the lungs highly permeable to antibodies; the Russian authors, like Fox earlier, were dealing with the passage of antibodies from the lumen of the respiratory tract into the blood stream. Even more to the point is the demonstration by Olitsky and Schlesinger (1941) that the dissemination of herpes virus was greatly enhanced by *mild* but not by severe inflammation. The facilitation of spread and the increase of tissue permeability by another of the substances used in our study (dicoumarin) was recently observed

by Thuerer and Angevine (1949); the effect was related to the lack of fibrinous blockade in the inflamed area.

In immune hosts such alterations of tissue permeability in the region to be infected are bound to lead to the local accumulation of antibody, and thereby to the enhancement of actual resistance. The importance of antibody concentration at the site of infection has been widely recognized, and in his admirable review of the subject Friedemann (1947) shows it up as the salient feature of immune reactions *in vivo*. Pathotopic potentiation of immunity, therefore, does not bring anything new in principle, merely in practice. Indeed, the literature contains several reports of immunological findings which, in retrospect, could be classified as pathotopic potentiation. The increased resistance of young heifers to *Tr. foetus* infection during pregnancy (Kerr and Robertson, 1946a, b; 1947), and especially the observations of Balozet (1947) hardly allow of any other interpretation. The latter author, when testing for anti-brucella agglutinins in goat's milk, found several times higher antibody titres in the milk coming from a compartment of the udder which had been affected by mastitis. His immediate conclusion, that serum and extravascular antibody titres are not strictly correlated, is identical with our thesis (Fazekas de St.Groth and Donnelley, 1950b) that influenza immunity is not conditioned by serum antibody titres but by the concentration of antibody at the site of virtual infection.

SUMMARY.

From diverse experiments performed with ten agents, half of which were effective in enhancing influenza immunity when given by the nasal route, it is concluded that:

(a) pathotopic potentiation is determined primarily by the response of the host organism, and only indirectly by the material properties of the adjuvant eliciting that response;

(b) the adjuvant has to act at the site of future infection;

(c) the underlying mechanism is essentially an increase in capillary and/or tissue permeability;

(d) only *mild* inflammation, with no demarcating fibrinous blockade, allows of the local accumulation of antibodies and thereby of enhancement of specific resistance.

REFERENCES.

Balozet, L. (1947): Bull. Ac. Vét. France 20, p. 204.
Fazekas de St.Groth, S. (1948a): Austral. J. exp. Biol., 26, p. 29.
Fazekas de St.Groth, S. (1948b): *Ibid.*, 26, p. 271.
Fazekas de St.Groth, S. and Donnelley, M. (1950a): *Ibid.*, 28, p. 45.
Fazekas de St.Groth, S. and Donnelley, M. (1950b): *Ibid.*, 28, p. 61.
Fazekas de St.Groth, S. and Donnelley, M. (1950c): *Ibid.*, 28, p. 77.
Fazekas de St.Groth, S., Donnelley, M. and Graham, D. M. (1951), *Ibid.*, 29, p. 323.
Fazekas de St.Groth, S. and Graham, D. M. (1949): *Ibid.*, 27, p. 83.
Fox, J. P. (1936a): J. Immunol., 31, p. 7.
Fox, J. P. (1936b): *Ibid.*, 31, p. 293.
Friedemann, U. (1947): Bact. Rev., 11, p. 275.
Halpern, B. N. and Roux, J. L. (1949): C. r. Soc. Biol., 143, p. 923.
Halpern, B. N. and Roux, J. L. (1950): Sem. Hôp. Paris 26, p. 1,806.
Henle, W. and Henle, G. (1946): J. exp. Med. 84, p. 639.
Kerr, W. R. and Robertson, M. (1946a): J. comp. Path., 56, p. 38.
Kerr, W. R. and Robertson, M. (1946b): *Ibid.*, 56, p. 101.
Kerr, W. R. and Robertson, M. (1947): *Ibid.*, 57, p. 301.
Olitsky, P. K. and Schlesinger, R. W. (1941): Science, 93, p. 574.
Parfentjev, I. A. (1950): Yale J. Biol., 23, p. 28.
Parfentjev, I. A. and Goodline, M. A. (1948): J. Pharm. exp. Ther. 92, p. 411.
Parfentjev, I. A. and Schleyer, W. (1949): Arch. Biochem., 20, p. 341.
Silber, L. A. and Baidakova, Z. L. (1945): Zh. Mikr. Epid. Immun., 4, p. 41 .
Stone, J. D. (1947): Nature, 159, p. 780.
Stone, J. D. (1948a): Austral. J. exp. Biol., 26, p. 49.
Stone, J. D. (1948b): *Ibid.*, 26, p. 287.
Thuerer, G. R. and Angevine, D. M. (1949): Arch. Path., 48, p. 274.

RECOMBINANT VIRUSES OBTAINED FROM DOUBLE INFECTIONS WITH THE INFLUENZA A VIRUSES MEL AND NEURO-WS

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In a series of papers recently published Burnet and Lind (1951a, b) it was shown that from the brains of mice inoculated intracerebrally with a mixture of two influenza viruses, a third type of virus could be isolated, presenting characters which appeared to derive from *both* original types. This was regarded as an example of "recombination" the word being used in the same sense as it is applied to corresponding phenomena with bacterial viruses.

The experiments all exploited the situation in which a neurotropic influenza virus strain of one serological type (WS) was mixed with a non-neurotropic strain of recognizably different character, e.g. MEL. The typical recombinant showed capacity to induce fatal infection by intracerebral inoculation, but had the serological character of the non-neurotrope.

The experiments reported were essentially of a preliminary character to establish that the phenomenon could be observed with regularity, and with several different non-neurotropic components. On returning to this type of investigation, our first objective was to analyse more closely the types of recombinant obtained from double infection with the neurotropic strain NWS and the standard non-neurotropic strain MEL.

The greater part of this paper is concerned with a single experiment in which all the characteristic features obtained in a large number of similar experiments could be observed. It will be shown that the recombinant strains obtained, have a relatively constant set of properties which clearly distinguish them from either "parent" strain, and in addition show a range of variation in other properties especially in their neurotropic capacity. Evidence will be given that a certain degree of serological change, not previously noted, is associated with recombination.

MATERIALS AND METHODS.

Viruses.

The strains used in most of the experiments were (1) the standard influenza A strain MEL isolated in 1935 and, after adaptation to mice by the intranasal route, maintained by passage in amniotic or allantoic cavities of chick embryos, (2) Stuart Harris's (1939) strain

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NWS, a neurotropic derivative of the classical WS strain of influenza A virus. Both strains were re-isolated from the stocks used in previous experiments by the method of limiting dilution in the allantoic cavity (Burnet and Lind, 1951a and b) and a number of ampoules of each fluid stored in a dry ice cabinet. For each experiment, a fresh ampoule was thawed out and mixtures prepared shortly before inoculation.

In some experiments other variants of MEL were used as the non-neurotropic component. These were the strain MEL-Egg, derived by chorioallantoic passage 15 years previously (Burnet, 1936; Burnet and Bull, 1944) and the three strains *Ms*, *Mr* and *m* recently described by Isaacs and Edney (1950a, b).

Mouse inoculations.

Mice of the Institute strain between 4 and 5 weeks old were inoculated intracerebrally under ether anaesthesia. Virus dilutions were made in saline containing 10 p.c. of normal horse serum and the volume inoculated was 0·03 ml. Symptoms and deaths were recorded and the survivors discarded at 14 days. In all experiments involving the use of 2 viruses, the required dilutions were mixed and a single 0·03 ml. inoculation given.

Isolation of virus from mouse brains.

At an appropriate time, usually 4 days after inoculation, mice were anaesthetized with ether and exsanguinated by cutting the subclavian artery. The brain was removed aseptically, ground with alundum abrasive and suspended in two ml. of saline. After light centrifugation serial dilutions, usually 10^{-1}, 10^{-2}, 10^{-3}, of the supernatant fluid were inoculated into the allantoic cavity of 11 day chick embryos, 4 eggs being used per dilution. In many experiments similar dilutions were also made in dilute immune serum from a ferret immunized by infection with NWS.

The fluids were tested for haemagglutinin after 2 days' incubation at 35° C., positive fluids were harvested for further study and eggs giving negative reactions were returned for another 24 hours' incubation. Any fluids developing haemagglutinin between the second and the third days were also harvested.

Characterization of virus in allantoic fluids.

As a routine each positive fluid was first titrated for its haemagglutinin content against fowl cells; incidentally the character of the deposited cells was noted as "slippery," S, when the agglutinated cells slipped readily to the centre of the tube and produced a non-granular suspension when shaken up (NWS type) or "firm," F, where a persisting "shield" of agglutinated cells was present and when resuspension of the cells in the saline gave a definite granularity visible with a low power lens (MEL type).

The serological type of the virus was determined by using fluid diluted to give 5 agglutinating doses for abbreviated antihaemagglutinin titrations of standard anti-MEL and anti-WS ferret immune sera. The starting dilutions of serum 1:500 and 1:100 respectively were high enough to avoid any nonspecific or cross neutralization. All strains isolated from mouse brains doubly infected with the two strains were unequivocally of one type or the other. Very rarely a mixed infection with almost equal titres of two viruses was detected by the fact that neither antiserum gave definite neutralization; but on each occasion the components were separated by a subsequent passage at limiting dilution.

A portion of the allantoic fluid was heated in a water-bath at 56° C. for 30 minutes, and subsequently titrated. Fluids infected with NWS lose all haemagglutinating power after such treatment. Fluids containing MEL show very little reduction in titre. The capacity of the heated virus to serve as an indicator of mucin inhibitor was then determined by testing 5 agglutinating doses against tenfold serial dilutions of ovomucin prepared according to the method of Gottschalk and Lind (1949). MEL is not converted into an indicator by simple

heating (Stone, 1949). As in the previous work those fluids which were converted into potent indicators were regarded provisionally as recombinant viruses. All were serologically MEL and are referred to as NM strains.

Except for special reasons further tests were made with "pure clone" virus obtained by titrating the primary fluid in tenfold dilutions and using the fluids from the limiting infective dilutions. If fluids from three eggs all showed the same reactions *in vitro* as the parent fluid, they were regarded as "pure clones" and one was used in tests for pathogenicity in mice and chick embryos.

The routine test for neurotropic activity was to inoculate 3 mice with allantoic fluid diluted 10^{-2} and 3 with a 10^{-4} dilution. If all six mice died within 7 days the result was graded as +++, if some deaths were delayed but not more than one mouse survived, ++, more than one survivor but some deaths, +, symptoms but no deaths, ±, and neither symptoms nor deaths, 0.

Pathogenicity for the chick embryo was tested by chorioallantoic inoculation of virus diluted usually about 10^{-3} in 12 day embryos. The embryos were examined after 3 days' incubation primarily for specific death of the embryo but the character of the foci on the chorioallantois was also noted.

Two other *in vitro* tests were used in some experiments. *Position in the receptor gradient* was determined by the use of fowl cells treated for a standard period, 30 minutes, with graded dilutions of RDE (Stone, 1947). The enzymic action was stopped with citrate and the cells washed and tested in 2 p.c. citrate saline against 5 agglutinating doses of each virus. Control strains of standard viruses were included.

Enzymic action on ovomucin was tested by the method described by Burnet (1949) in which 50 haemagglutinating doses of virus are allowed to act for 18 hours at 37° C. on a standard amount of ovomucin in saline, buffered at pH 6·2 with acetate and containing 0·1 p.c. of $CaCl_2$. After heating for 30 minutes at 65° C. to destroy the haemagglutinin, the residual inhibitor was titrated against the indicator viruses H-LEE and T-MEL (Stone, 1949).

EXPERIMENTAL.

Preliminary experiments.

All the experiments to be reported were carried out using a single fluid virus of NWS stored in ampoules in a solid CO_2 refrigerator, and for all those in which MEL was used a single stock of that virus was similarly maintained. Several modifications of the original technique were tested to find the conditions for the best yield of recombinant strains. It was finally determined that the optimal mixture to inoculate was one containing undiluted MEL virus and a 1:10 dilution of NWS in equal volume, and the best time to harvest the brain, four days after inoculation. At this time, one often isolates an occasional MEL strain, but the yield of NM strains is better than at 5 days. There is active antibody production between 4 and 5 days, and it is probable that this is an important factor in diminishing the yield.

On the assumption from limited experiments made in our previous work that an anti-NWS ferret serum which had no action *in vitro* or *in ovo* on MEL would be equally inert against NM strains, this was used in the early experiments to inactivate surviving NWS. It gradually became evident, however, that when brains were ground and emulsified in saline containing 10 p.c. of immune ferret serum, the yield of NM strains was much lower than if an inert diluent was used. With the standard inoculum and the time of harvesting, no difficulty was experienced by overgrowth of NWS.

The main objective of the present experiments was to obtain and study in detail a large number of strains of virus from double infections, in the hope of determining finally whether a recombination process was the only possible interpretation of the appearance of NM forms.

To avoid repetition, details will be given only of a single experiment from which about half of the strains closely examined were derived.

TABLE 1.

Analysis of mouse brain emulsion 72618–20 from brains harvested 4 days after inoculation with MEL (undiluted) and NWS (10^{-1}).

	Diluent	Dilution	Strains isolated
A. First titration	NHSS	10^{-3}	NM[1] NM[2] NWS
		10^{-2}	NM[3] NM NM NWS
		10^{-1}	NM[4] NM NM M
	F361/10	10^{-3}	— — — —
		10^{-2}	+ — — —
		10^{-1}	NM[5] NM — —
B. Second titration	NHSS	10^{-4}	18 —
		$10^{-3.5}$	5 NM, 1 NWS, 14 —
		10^{-3}	8 NM, 2 M*, 1 NWS, 8 —

1, 2, 3, 4, 5. Strains from first titration studied in detail.

\+ Virus isolated, but titre too low for study.

* One M fluid was subsequently shown to be a mixture of M and NM.

TABLE 2.

Mouse pathogenicity of strains re-isolated by the method of limit dilution.

NM strain	Fluid	Mice Intracerebral 10^{-2}	Mice Intracerebral 10^{-4}	Grading
1	70804	S S S	S S S	—
2	70540	5 12 14	S S S	+
3	70557	6 6 6	5 6 6	+++
4	70846	7 7 8	8 8 8	++
5	70864	5 7 7	6 6 6	+++

S: mouse survived 14 days. 5, 6, etc.: mouse died with typical symptoms 5, 6, etc., days after inoculation.

Analysis of a typical mouse brain emulsion.

Mice were inoculated intracerebrally with the standard MEL-NWS 10^{-1} mixture and three of them killed about 92 hours later. The brains were ground with alundum abrasive powder and suspended in 2 ml. of saline per brain. The supernatant fluids were pooled and lightly centrifuged to get rid of cellular debris. The bulk of the supernatant fluid was ampouled for storage in solid CO_2, and from the rest serial 10-fold dilutions were made (1) in normal horse serum saline, (2) in anti-NWS ferret serum F361 diluted 1:10 in normal saline. Four 11-day embryos were inoculated allantoically with each dilution and tested for haemagglutinin after 2 days' incubation at 35° C. Positive fluids were harvested and negative eggs returned for a further test after 24 hours' more incubation (Table 1).

The fluids obtained were characterized by the series of tests described in the section on methods, and 5 of those giving NM reactions subjected to re-isolation by limit dilution. The re-isolated viruses all gave NM reactions and were tested intracerebrally in mice. The

results are given in detail in Table 2 to show the principle used in grading the pathogenicity of strains for mice by the intracerebral route.

A fortnight later an ampoule of the same brain emulsion was thawed and dilutions of 10^{-3}, $10^{-3.5}$ and 10^{-4} prepared to obtain directly limit dilution clones of virus. Each was inoculated into the allantoic cavity of twenty 11-day embryos. The results are shown in Table 1, dead embryos being omitted.

These fluids were put through the whole range of tests without further purification in order to avoid any possible changes resulting from an additional egg passage. As might be expected from the figures for the 10^{-3} dilution one fluid showed a double infection giving the *in vitro* characters of unmodified MEL but proving pathogenic for mice intracerebrally. From the brains a typical NM strain was obtained. All 13 NM strains shown in Table 1B with the exception of one contaminated fluid were fully tested, and in Table 3 the results with 6 representative strains are shown in contrast to one strain each of MEL and NWS isolated from the same brain emulsion. In Table 3 the first two lines show the characteristics of the two parent strains MEL and NWS. It will be observed that there are significant and often very striking differences in regard to all 10 criteria used. The reactions shown are typical of those obtained with many other fluids infected with one or other of these strains.

TABLE 3.

Virus type	Fluid No.	1. Type of H.A.	2. H.A. titre	3. H.A. titre 56°	4. Anti H.A. MEL	5. Anti H.A. NWS	6. Indicator state	7. Enzymic activity	8. Receptor gradient position	9. Mice Intracerebral	10. Lethality C.A.
MEL	71024	F	750	400	6,000	< 100	20	< 50	2,500	—	0
NWS	70995	S	140	< 10	< 500	1,300	—	10,000	3,500	+++	3
NM	70994	F	430	80	3,000	< 100	10,000	10,000	1,500	+++	2
	70998	F	240	120	5,000	< 100	5,000	8,000	1,500	+++	3
	71003	F	430	120	3,000	< 100	10,000	10,000	1,200	+	3
	71007	F	290	100	3,500	< 100	12,000	7,000	1,500	+++	2
	71015	F	290	60	3,000	< 100	10,000	15,000	1,200	—	2
	71019	F	480	100	3,000	< 100	10,000	20,000	1,200	±	1

Notes: 1. Type of haemagglutination: F, firm shield; S, slippery deposit.

2. Haemagglutinin titre against fowl cells.
3. Haemagglutinin titre after heating at 56° C. for 30 minutes.
4, 5. Antihaemagglutinin titres of anti-MEL and anti-NWS ferret sera against 5 agglutinating doses of the strain.
6. Inhibitory titre of ovomucin against 5 agglutinating doses of virus heated as undiluted allantoic fluid to 56° C. for 30 minutes.
7. Level to which the inhibitory titre of ovomucin against indicator-MEL virus is reduced by treatment with 10 agglutinating doses of virus. Control titre 20,000.
8. Position in receptor gradient. Dilution of RDE needed to reduce agglutinability of fowl cells to "+" for 5 agglutinating doses of virus.
9. Intensity of infection following intracerebral inoculation (see Table 2).
10. Number of embryos dying with specific lesions after chorioallantoic inoculation. Three embryos tested with $10^{2.5}$ dilution of each strain.

The qualities of the NM strains, i.e. those strains isolated from mixedly infected brains, which were serologically MEL but were convertible to indicator by simple heating, can best be demonstrated by successive consideration of each of the 10 differentiating criteria:

(1) The pattern of agglutinated cells differs sharply between MEL and NWS, the former giving a firm shield over the bottom of the tube and when the cells are gently re-suspended, granularity is clearly visible with a hand lens. Agglutination by NWS is slippery,

and the cells on gentle shaking, produce a smooth non-granular suspension. NM strains are all of the MEL type.

(2) MEL strains give high titre fluids with haemagglutinin titre around 1,000, NWS fluids are usually between 80 and 240, while with one exception, the NM fluids give values between 240 and 480.

(3) On heating at 56° C., MEL strains show less than a 50 p.c. drop in haemagglutinin titre, NWS are always completely inactivated. NM strains are intermediate, the titre usually being ⅓ or ¼ that of the unheated fluid.

(4) and (5) Antihaemagglutinin titres of the standard ferret sera anti-MEL (F358) and anti-NWS (F360) indicate a consistent difference between MEL and NM on the one hand, and NWS on the other. The NM strains show no evidence of any WS component.

(6) MEL is never converted to indicator by simple heating at 56° C., and NWS is inactivated completely. All NM strains are consistently converted to indicator, this quality being used to define them.

(7) NWS is an extremely weak enzyme, MEL rapidly destroys ovomucin inhibitor for the corresponding indicator T-MEL. NM strains vary slightly from one to another, but resemble NWS in having very poor enzymic action on ovomucin.

(8) All strains are in the early section of the receptor gradient, and there are inconsistencies from one fluid of a given strain to another. In general, the NM strains are further to the right (lower numerical value) than either NWS or MEL.

(9) The variability in intracerebral pathogenicity from one NM strain to another has already been stressed. Of 32 distinct strains of NM isolated from mixed infection, the distribution according to intensity of action is as follows: 10+++, 9++, 5+, 2±, 6—.

(10) With one exception, all NM strains resembled NWS in producing well defined foci on the chorioallantois of 12 day embryos, and in killing some or all of the embryos by the 3rd day with the characteristic specific lesions, the most notable of which is massive haemorrhage in the brain. MEL produces tiny foci and does not kill the embryo.

Recombination between the MEL variant Ms and NWS.

The strain *Ms* was derived by Isaacs and Edney (1950) from MEL by growth in the presence of homologous immune serum. The only character in which it differs from MEL is in its resistance to neutralization by immune serum anti-MEL. This is best shown in the allantoic cavity, but is also evident in antihaemagglutinin tests. With our standard anti-MEL serum giving a titre averaging 4,000 with MEL, the titre against *Ms* averages 900. In an experiment entirely comparable to those described except for the replacement of MEL by *Ms*, mice killed at 4 days gave two strains like *Ms* and 10 which resembled NM in being convertible to indicators by simple heating, but showed the low neutralizability by anti-MEL serum characteristic of the parent strain. These can be called *NMs* strains. Three *NMs* and one *Ms* fluids were titrated and the limit dilution fluids tested in mice. The *NMs* fluids gave +++, +++, + gradings, the *Ms* strain was inert. The second and third of the *NMs* strains tested on the chorioallantois produced typical fatal infections, the parent *Ms* produced powdery foci with no effect on the embryo.

In brief, the results were identical with those obtained with MEL, except for the characteristically reduced neutralization of both the parent and the derivative by anti-MEL serum.

Limited tests with two other MEL variants, the "Melbourne-egg" strain described by Burnet (1936) and Isaacs' and Edney's 1950 strain *m*, failed to give any types other than the originals from mixed infections.

An atypical NWS strain from mixed infection.

In all experiments a watch was kept for strains serologically WS which might show one or other MEL characters. Two fluids from different experiments were observed, which gave firm shield agglutination, but were serologically NWS. One of these, 70175, showed no other

differences from NWS, the second, 70025, showed other interesting characteristics. Its infective titre ($10^{7.8}$) and haemagglutinin titres (380, 320) in allantoic fluid were unusually high, but the haemagglutinin titre was destroyed as usual at 56° C. Its enzymic activity against ovomucin was tested in comparison with NWS by Stone's (1949) method using H-LEE as indicator. The relation between haemagglutinin titre and inhibitor-destroying titre was, for two fluids derived from 70025, 380/240, 320/420; for a normal NWS 160/20. Unlike normal NWS, the allantoic fluids infected with the abnormal strain contained no persisting inhibitor for H-LEE, another evidence of increased enzymic action.

Receptor gradient tests showed a sharp shift to the right. Using the same method of expressing the position in the gradient as in Table 3, the following values were obtained:

MEL. 1,800. 4 strains of NWS including 700175 > 2400.

Three strains derived from 70025. 1,000, 1,200, 1,200.

The figures represent the reciprocal of the dilution of RDE giving cells which are agglutinated to the + level by 5 agglutinating doses of the virus concerned.

The pathogenicity of 70025 derivatives for mice intracerebrally and intranasally and for chick embryos by the chorioallantoic route was identical with that of other NWS strains.

In summary, this strain showed three characters approaching those of MEL, one, its position in the receptor gradient, which was unique, and five corresponding to NWS.

DISCUSSION.

The results reported above confirm and extend the findings of Burnet and Lind (1951) that from mixed infections of the neurotropic influenza A strain NWS with another non-neurotropic strain MEL or *Ms*, well characterized virus strains differing sharply from the two original types can be obtained.

The most direct explanation of the phenomena is that already put forward (Burnet and Lind, 1951) that the new type NM or *NMs* is a recombinant analogous to those produced in double infections by related bacterial viruses (Delbrück and Bailey, 1946).

In the bacterial virus experiments it is possible to obtain from a single mixedly infected bacterium the whole progeny and show for instance that infection by phages having markers A^1B^1 and A^2B^2 results in 30 p.c. A^1B^1 30 p.c. A^2B^2 20 p.c. A^1B^2 and 20 p.c. A^2B^1. In our experiments with influenza viruses it is only possible to obtain those variants or recombinants which can survive for four days in the complex environment of the mouse brain and can subsequently multiply in the allantoic cavity of the chick embryo. Further, only those virus types are detectable which are present in the mouse brain emulsions in greater numbers than any other type or at least are so nearly equivalent in number to the dominant form that examples can be obtained by the use of a practicable number of chick embryos. Direct proof of recombination by obtaining reciprocal "hybrids" is therefore not possible.

The capacity of influenza viruses to undergo mutation is well known and by various procedures far-reaching changes in quality have been obtained. The obvious question therefore arises as to whether our NM strains are not direct mutants of MEL, rather than recombinants combining characters derived both from MEL and NWS.

The simplest form in which this alternative hypothesis can be framed is that in any population of MEL including necessarily a "pure clone" fluid, there appear a proportion of NM mutants. These, however, can never be detected by any straightforward method because of interference by the dominant MEL form both in the mouse brain and in the chick embryo. In the presence of an established NWS infection in the mouse brain there is provided a highly selective environment which allows the NM mutants to overgrow both NWS and MEL. Strictly speaking, one might equally well assume that NM may be a mutant of NWS which, unlike the original form, is not interfered with by MEL and so finds an opportunity for selective survival in mixed infections.

It is difficult to devise an experimental proof or disproof of this hypothesis and it can be discussed only on the basis of probabilities. If NM variants arise in all MEL fluids one must assume that they are also prevented from manifesting their action in chick embryos as well as in the mouse brain as they all have the quality of producing general haemorrhagic lesions in the chick embryo. These are never seen with MEL or with the mutants *Ms Mr* and *m*. In view of the general experience that with the partial exception of the O-D change adaptation in influenza and other viruses is a process of change by small steps, the appearance in one stage of all the NM types seems almost infinitely unlikely.

If the mutant is not present in the fluid inoculated the only other possibility is that it arises from MEL in the mouse brain under the directive influence of some mutagenic agent produced by the interaction of NWS and brain cells. There are no biological analogies for such action by any means short of the transfer of genetic material from one organism to another.

TABLE 4.

Distribution of 10 marker characteristics in 4 derivative strains.

	Marker characteristic									
Strain	1	2	3	4	5	6	7	8	9	10
NM 70998	M	I	I	M	M	U	N	U	N	N
NM 71015	M	I	I	M	M	U	N	U	M	N
NWS 70025	M	I	N	N	N	N	I	U	N	N
NWS 70175	M	N	N	N	N	N	N	N	N	..

For significance of symbols see text. .. not tested.

Quite apart from the difficulties of any alternative hypothesis there is a great deal of positive evidence in favour of the recombinant hypothesis. In Table 3 and in comments in the text we have taken 10 qualities numbered consecutively in which MEL and NWS can be shown to differ consistently. They can therefore be regarded as 10 genetic markers. We may tabulate the

qualities of NM or other alleged recombinants as M when they correspond to those of MEL, N when they correspond to NWS, I when they are intermediate and U when they are unique, i.e. not found in either MEL or NWS. Two NM strains and the two aberrant NWS strains described earlier may be tabulated in this fashion. (Table 4).

It should be recognized that the two unique qualities of NM strains, the conversion to indicator on simple heating and the shift to the right in the receptor gradient are both shown by other variants of WS and may well represent qualities derived from NWS though not shown in the phenotype. If this is accepted then all the points in which NM differs from MEL represent approaches to NWS character. Similarly but in much less striking fashion the two aberrant WS strains approach M in those characters in which they deviate from the normal form.

We are inclined to lay particular stress on marker 10 the capacity to produce lethal haemorrhagic lesions in chick embryos. This is correlated with the NM *in vitro* characters not with pathogenicity for the mouse brain. Adaptation to growth in the mouse brain cannot be assumed therefore to have automatically conferred this other type of pathogenicity on the NM strains. Further, we have shown that a MEL variant "Melbourne-egg" with high lethality for the chick embryo has no power to infect mice intracerebrally. It seems impossible to explain this quality of NM except by deriving it from NWS.

Our conclusion therefore is that these experiments establish the existence of a process of recombination by which some of the qualities of two different strains of influenza virus can be transferred to a single descendant virus. The mechanism of the process remains to be elucidated. The present results are compatible with a mechanism similar to that postulated by Luria (1950) for the multiplication of bacterial viruses.

SUMMARY.

From mixed infections in mouse brains of the neurotropic influenza strain NWS and the non-neurotropic strain MEL, virus strains which differ sharply in character from either of the originals can be obtained.

A large number of such "NM" strains have been studied in regard to 10 criteria which differentiate clearly between the two original types. These strains are fairly homogeneous in character, a typical example showing 3 MEL qualities, 3 corresponding to NWS, 2 intermediate qualities and 2 not found in either original. They show, however, a wide range of differences in pathogenicity for the mouse brain.

It is concluded that these strains arise by a process of recombination analogous to that observed with bacterial viruses.

REFERENCES.

Burnet, F. M. (1936): Brit. J. exp. Path., 17, p. 282.
Burnet, F. M. (1949): Austral. J. exp. Biol., 27, p. 361.
Burnet, F. M. and Bull, D. R. (1944): *Ibid.*, 22, p. 173.
Burnet, F. M. and Lind, P. E. (1951 a and b): J. gen. Microbiol., 5, p. 59, 67.
Delbrück, M. and Bailey, W. T. (1946): Cold Spring Harbour Symp. Quant. Biol., 11, p. 33.
Gottschalk, A. and Lind, P. E. (1949): Brit. J. exp. Path., 30, p. 85.
Isaacs, A. and Edney, M. (1950 a and b): *Ibid.*, 31, p. 196, 209.
Luria, S. E. (1950): Science, 111, p. 507.
Stone, J. D. (1947): Austral. J. exp. Biol., 25, p. 137.
Stone, J. D. (1949): *Ibid.*, 27, p. 337.
Stuart-Harris, C. H. (1939): Lancet, i, p. 497.

PURIFICATION OF AUSTRALIAN STRAINS OF COXSACKIE VIRUS.

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In the course of studying the properties of several viruses pathogenic for suckling mice (Stanley and Hayes, 1950; Stanley *et al.*, 1951), it was found necessary to attempt to purify certain strains to determine if virus, free from large amounts of non-virus protein, could be used in haemagglutination or complement-fixation tests. This paper describes the purification procedures adopted with three strains. The use of these purified preparations in complement-fixation and haemagglutination tests will be the subject of a further communication.

Materials.

Source of virus. The strains employed for this investigation were W32 and S4 (myositis-producing strains) and E46, which produces mainly encephalitis in suckling mice. These three strains were shown to be serologically dissimilar (Stanley *et al.*, 1951). Freshly harvested tissues of suckling mice paralysed and dying from infection with the viruses, provided the materials from which the viruses were purified.

EXPERIMENTAL.

Two methods of purification were used, viz:

Method I. This involved the use of protamine sulphate to remove non-virus protein (Warren *et al.*, 1949) and subsequent ultracentrifugation to sediment the virus. The technique as carried out with the W32 strain was as follows: 110 gm. of infected tissue were ground in sand with 200 ml. of cold distilled water. Penicillin and streptomycin were added to give concentrations of 100 units/ml. and 0·5 mg./ml. respectively. The suspension was frozen for 18 hours at —23° C. and then allowed to thaw. The precipitate was cleared by centrifugation at 5,000 r.p.m. for 15 minutes at 4° C., and the volume adjusted to 300 ml.

To each 100 ml., 0·5 gm. protamine sulphate powder was added with stirring until dissolved. The material was kept at 4° C. for 1 hour, with stirring every ten minutes. The precipitate resulting from the addition of protamine was removed by centrifugation at 5,000 r.p.m. for 20 minutes at 4° C. The clear supernatant fluid containing virus and residual protamine was recovered. This supernatant fluid was then placed in the No. 40 rotor of a Spinco ultracentrifuge and the virus material sedimented at 40,000 r.p.m. at 4° C. for 1½ hours.

The supernatant fluid was recovered for analysis. The deposit was recovered in 30 ml. of the supernatant and frozen at —23° C. for 18 hours. The material was thawed and centrifuged at 40,000 r.p.m. at 4° C. for 2 hours. The deposit was taken up in isotonic phosphate buffer and diluted to 100 ml.

Method II. This technique was used for all three virus strains. Batches consisting of 60 gm. to 240 gm. of tissue were put through at a time. Distilled water extracts comprising 50 p.c. of tissue were made by mincing for sixty seconds in a chilled Waring-blendor sealed at the top with vaselined cellophane. The blendor was allowed to remain at 4° C. for 1 hour before the fluid was decanted. Penicillin and streptomycin were added to give concentrations of 100 units/ml. and 0·5 mg./ml. respectively. The suspension was frozen at —23° C. for 18–24 hours, then allowed to thaw, after which it was cleared by centrifugation at 4° C. at 7,000 r.p.m. for 1 hour in the No. 20 rotor of the ultracentrifuge. The supernatant fluid was frozen for 48 hours at —23° C., after which it was allowed to thaw at room temperature. The resulting precipitate was removed by centrifugation at 4° C. at 7,000 r.p.m. for 1 hour in the No. 20 rotor. The supernatant fluid was removed and shaken with an equal volume of diethyl ether. The aqueous layer was recovered and excess ether removed *in vacuo*. The material was again frozen at —23° C. for 48 hours, after which time it was thawed and again cleared by centrifugation. The clear fluid was then placed in the No. 40 rotor of the ultracentrifuge and spun at 40,000 r.p.m. at 4° C. for 3 hours. The pellets were removed and ground in buffered saline (phosphate buffer pH = 6·4 plus an equal volume of 0·85 p.c. NaCl). The product was kept at —23° C. in 10 ml. volumes.

Four samples were removed for testing from each preparation. These are referred to as A, B, C and D in Table 1. Sample A represents the original tissue suspension from which debris and coarse particles have been removed by light centrifugation. Sample B represents the supernatant fluid after treatment with protamine sulphate (method I) or after freezing and thawing followed by ether extraction (method II). Sample C represents the supernatant fluid recovered from the ultracentrifuge tubes after sedimentation of the virus at high speed, while Sample D refers to the suspensions made from the pellets formed during ultracentrifugation. Quantitative chemical tests for protein nitrogen and a Molisch test were made on each sample. The infective dose 50 (ID_{50}) was determined by intraperitoneal inoculation of ten-fold dilutions into litters of suckling mice.

TABLE 1.

	Virus	Fraction	Volume (ml.)	Mg. nitrogen/ml.	ID_{50}	Molisch test
Method I.	W32 Passage 12	A 30 p.c. tissue suspension	300	2·29	$10^{-5.0}$	Strong
		B protamine sulphate supernatant	300	1·64	$10^{-4.4}$	Strong
		C ultracentrifuge supernatant	300	1·57	$10^{-2.8}$	Strong
		D ultracentrifuge deposit	300	0·01	$10^{-4.2}$	Negative
Method II	S4 Passage 10	A 40 p.c. tissue suspension	500	2·10	$10^{-8.1}$	Strong
		B supernatant after ether extraction	500	0·82	$10^{-7.0}$	Strong
		C ultracentrifuge supernatant	500	0·73	$10^{-4.5}$	Strong
		D ultracentrifuge deposit	500	0·09	$10^{-6.5}$	Faint
	S4 Passage 14	A 40 p.c. tissue suspension	300	1·98	$10^{-8.8}$	Strong
		B supernatant after ether extraction	300	0·80	$10^{-7.1}$	Strong
		C ultracentrifuge supernatant	300	0·69	$10^{-3.3}$	Strong
		D ultracentrifuge deposit	300	0·05	$10^{-7.0}$	Weak
	E46 Passage 9	A 40 p.c. tissue suspension	200	2·08	$10^{-7.4}$	Strong
		B supernatant after ether extraction	200	1·25	$10^{-7.2}$	Strong
		C ultracentrifuge supernatant	200	1·08	$10^{-2.8}$	Strong
		D ultracentrifuge deposit	200	0·09	$10^{-6.2}$	Faint

DISCUSSION.

It is clear that a large amount of non-virus protein may be removed either by protamine sulphate or by alternate freezing and thawing. Active virus material was lost in both procedures. Although Warren *et al.* (1949) do not refer to Coxsackie viruses in their study on the purification of viruses by protamine sulphate, it is obvious from the experiments outlined above that a modification of their method without the use of heparin may be used in the purification of these viruses. Most of the virus, but not all, could be recovered from the clear, light brown pellet formed by ultracentrifugation. This observation is in keeping with the findings of Melnick *et al.* (1949) and of Quigley (1949), whose ultrafiltration and ultracentrifugation experiments have revealed that one of the group A viruses (Dalldorf, 1950) has a particle size of less than 10 μm.

SUMMARY.

The purification of three serological types of Australian strains of Coxsackie virus is described. Virus material was concentrated by ultracentrifugation.

REFERENCES.

Dalldorf, G. (1950): Bull. N.Y. Acad. Med., 26, p. 329.
Melnick, J. L., Shaw, E. W. and Curnen, E. C. (1949): Proc. Soc. exp. Biol. Med., 71, p. 344.
Quigley, J. J. (1949): Proc. Soc. exp. Biol. Med., 72, p. 434.
Stanley, N. F. and Hayes, L. (1950): Austral. J. Sci., 13, p. 81.
Stanley, N. F., Hayes, L. and Dorman, D. C. (1951): In press.
Warren, J., Weil, M. C., Russ, S. B. and Jeffries, H. (1949): Proc. Soc. exp. Biol. Med., 72, p. 662

SUCKLING MOUSE VIRUSES IN NEW SOUTH WALES; THEIR RELATIONSHIP TO THE COXSACKIE GROUP OF VIRUSES

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It has now been well established on the North American continent that viruses pathogenic for suckling mice may be isolated from the faeces of patients with the symptoms of poliomyelitis (Dalldorf and Sickles, 1948); Dalldorf, Sickles and Plager, 1949; Melnick, Shaw and Curnen, 1949; Curnen, Shaw and Melnick, 1949). The viruses differ serologically from the poliomyelitis virus and fail to cause the lesions characteristic of poliomyelitis when inoculated into monkeys. The properties peculiar to virus strains first reported were their ability to cause (1) paralysis and death in suckling mice and hamsters, but not in older animals, and (2) widespread destruction of striated muscle without evidence of histological changes in the central nervous system or cardiac muscle. The year following these observations, reports appeared of further isolations and studies of suckling mouse viruses (Howitt, 1950), which became known as the "Coxsackie" group of viruses after the town in New York State where the original isolations were made (Dalldorf, 1949). These preliminary studies showed that different serological types existed (Sickles and Dalldorf, 1949), and that they were widespread throughout certain parts of U.S.A., and could be isolated from sewage and flies (Melnick, Shaw and Curnen, 1949). Dalldorf (1950a) then revealed that it was possible to divide the strains thus far isolated into two groups (A and B) depending on the morbid anatomy of infection. Group A viruses produced degeneration of the skeletal muscle, while Group B strains produced mainly encephalitis.

Casals, Olitsky and Murphy (1949), Howitt and Benefield (1950) and Manire, Sulkin and Farmer (1950) demonstrated that complement-fixation tests could be used for the serological typing of Coxsackie strains and for detecting antibodies in human sera. Immunological reactions of the group were studied in greater detail at Yale University (Melnick and Ledinko, 1950; Kraft and Melnick, 1950; Melnick, Clarke and Kraft, 1950), and these indicated the reliability of the neutralization test and the importance of careful preparation of antigens for the complement-fixation test. Type-specific immunity could be conferred on young suckling mice through antibodies in the colostrum and milk of vaccinated mothers. It was further shown that strains of Coxsackie viruses could be typed by challenging the young mice of mothers vaccinated with known types of virus.

Because both poliomyelitis viruses and Coxsackie viruses have been isolated from stools of patients with paralysis during epidemics (Melnick and Kaplan, 1950; Armstrong *et al.*, 1950), the importance of the association of the two viruses in poliomyelitis cannot be overlooked, especially as antibodies to both viruses may develop simultaneously in a patient's serum.

On the other hand, observations (Huebner *et al.*, 1950) on the natural occurrence of the virus in a small community (free from clinical poliomyelitis) in Southern Maryland, indicate that caution should be exercised in assessing the significance of such a relationship. With the exception of the Maryland studies, most isolations of Coxsackie viruses have been made from patients with syndromes resembling paralytic poliomyelitis, non-paralytic poliomyelitis, aseptic meningitis, "summer grippe," pleurodynia (Bornholm disease), influenza, pyrexias of unknown origin and, more recently, herpangina (Curnen, Shaw and Melnick, 1949; Melnick, Clarke and Kraft, 1950; Weller, Enders, Buckingham and Finn, 1950; Findlay and Howard, 1950; Curnen, 1950; Huebner *et al.*, 1951).

It has thus become of importance to determine whether viruses similar to the Coxsackie group occur in Australia, and, if so, the part they play in the aetiology of poliomyelitis-like diseases, their natural occurrence in epidemic and inter-epidemic periods and the distribution of immune bodies in the population. We recently reported (Stanley and Hayes, 1950) the isolation of three virus strains from patients with "non-paralytic poliomyelitis." The following report describes in more detail the isolation and properties of seven viruses pathogenic for suckling mice. Five viruses were isolated from faeces and were shown to resemble the Coxsackie viruses of the American workers, while the remaining two viruses, isolated from throat washings, proved to be strains of herpes simplex.

Materials and Methods.

Selection of cases and material.

With the exception of the strain from sewage, the viruses were isolated from the faeces or throat washings of cases admitted to the Prince Henry Hospital with a provisional diagnosis of "poliomyelitis" during October and November of 1950. This period was just a few weeks prior to the highest incidence of poliomyelitis in the 1950–51 epidemic in New South Wales. Twenty consecutive cases were selected without knowledge of final diagnosis, but the histories of only those who gave positive virus isolations will be recorded here. The final diagnoses given refer to those made by the medical officer in charge of the case and without knowledge of the laboratory findings recorded in this paper.

Case 1. P29 virus. Final diagnosis: Bornholm disease. The patient was a male, aged fifteen, who was admitted to hospital four days after the onset of symptoms. He complained of headache, nausea, anorexia, and intermittent vomiting for four days. There was moderate neck rigidity and abdominal pain in the region of the umbilicus. He developed weakness of arms and legs for a few days, but this was absent eight days after admission. Biochemical, bacteriological and cytological examination of C.S.F. collected on admission revealed nothing abnormal. The patient was discharged twelve days after admission. The virus was isolated from throat washings.

Case 2. W32 virus. Final diagnosis: Bornholm disease. The patient was a male, aged twenty-three years, who was admitted to hospital six days after the onset of the following symptoms: headache, vomiting, stiff neck and severe epigastric pain. No muscular weakness could be detected. The C.S.F. collected the day before admission showed 65 lymphocytes/cmm.; protein 200, chlorides 740, and sugar slightly increased. The patient was discharged eight days after admission. The virus was isolated from faeces.

Case 3. J33 virus. Final diagnosis: acute anterior poliomyelitis. The patient was a male, aged seven years, who was admitted to hospital two days after the onset of symptoms. The patient was vomiting, had a sore back and neck, moderate neck rigidity, and showed slight weakness and loss of energy. The C.S.F. collected on admission showed 290 lymphocytes per cmm. Culture was sterile. Biochemical examinations were not performed. The patient

was transferred to another hospital fifteen days after admission. Slight residual muscle weaknesses were observed five months after onset. The virus was isolated from faeces.

Case 4. J37 virus. Final diagnosis: acute anterior poliomyelitis. The patient was a female five years old, who was admitted to hospital two days after onset of symptons—a cold in the head, headache, pain in the lumbar region, anorexia, pyrexia and a sore throat. Shortly after admission neck rigidity developed with opisthotonos and a positive Kernig's sign. Four days later there was no neck rigidity and no sign of muscle weakness. C.S.F. collected on admission showed 180 leucocytes/cmm., consisting of 85 p.c. polymorphs and 15 p.c. lymphocytes. Biochemical examination revealed sugar 53, chlorides 745 and protein 100. The patient was discharged eighteen days after admission with no signs of muscle weakness. The virus was isolated from faeces.

Case 5. E46 virus. Final diagnosis: mumps. The patient was a male, aged seven, who twelve days prior to admission to hospital had received a blow on the head and was unconscious for a short time. Three days later he developed pain in the left thigh and behind the left knee and complained of a headache. Eleven days after the blow the child showed slight neck stiffness. Lumbar puncture at this time revealed 15 lymphocytes/cmm. and protein 30. On admission to hospital, one day later, the child showed no neck rigidity and Kernig's sign was negative. Nine days after admission the child developed left parotid swelling. He was discharged ten days after admission as a case of mumps to be nursed at home. The virus was isolated from faeces.

Case 6. B48 virus. Final diagnosis: acute anterior poliomyelitis. The patient was a female, aged fourteen. She was admitted to hospital three days after complaining of pains in the legs, arms and neck. On admission all symptoms were gone except pain in the neck. Five days after admission slight weakness of the left gluteal muscle was noted. C.S.F. collected on admission revealed 200 leucocytes/cmm. consisting of 95 p.c. lymphocytes and 5 p.c. polymorphs. Biochemical examination showed globulin increased, protein 100, sugar 54, chlorides 745. The patient was discharged eighteen days after admission with no residual weakness. The virus was isolated from throat washings.

Sewage 4. The virus designated "Sewage 4" was obtained from a sample of raw sewage from a district (Herne Bay) in which a number of "poliomyelitis" patients had been resident. All patients made an uneventful recovery.

Concentration of virus from throat washings and faeces.

This was carried out as described in an earlier publication (Stanley and Hayes, 1950). Neo-peptone-heart broth (100 ml. volumes) was used for the throat washings, which were frozen generally within an hour of collection. In a few cases the virus was recovered from the entire 100 ml. by first spinning at 17,000 r.p.m. for 30 minutes in the No. 20 rotor of a Spinco ultracentrifuge, and later concentrating the virus from the supernatant by spinning for 1 hour at 40,000 r.p.m. in the No. 40 rotor. The deposit was taken up in 2 ml. of the supernatant fluid. All centrifugation was carried out at 4° C. Throat washings, faeces and the ultracentrifuge deposits of both were frozen until examination for poliomyelitis viruses could be carried out. Sera, collected when possible from cases during the acute and convalescent stages, were kept frozen at —23° C. without the addition of preservative, and after inactivation at 56° C. for 30 minutes.

Mice.

White mice that had been close-bred in this laboratory for ten years without the introduction of an outside strain were used throughout this work. Tests made for the presence of latent encephalomyelitis virus in the herds were consistently negative over the four-year period prior to the commencement of these studies. The suckling mice used for the original isolations, titrations, neutralization and heat susceptibility tests were less than 48 hours old. The average litter number of the first 500 litters used in these experiments was 8. In suck-

ling mice the intraperitoneal inoculum was 0·05 ml. and the intracerebral inoculum 0·01 ml. In adult mice the intracerebral inoculum was 0·03 ml.

Examination of mice.

Mice were checked twice daily from Monday to Friday, and once only on Saturdays and Sundays. The mice were handled with forceps which were flamed between handling each litter.

Preparation of immune sera.

Adult mice were inoculated twice weekly for three weeks by the intraperitoneal route with 0·2 ml. of 10 p.c. muscle suspensions of paralysed and dying mice. A batch of 30 mice was used for each virus, and these were bled from the heart one week after receiving the last injection. The serum was inactivated at 56° C. for 30 minutes before storing at —23° C.

Isolation of virus and preparation of suspensions.

Cultures for contaminating bacteria were made as a routine from the throat washings and faeces ultracentrifuge deposits as well as from the suspensions of brain and muscle made from suckling mice on primary isolation. Material was set aside for histological examination from each passage. Muscle, brain and some individual organs were always selected and in quite a number of cases the suckling mice were fixed intact and cut longitudinally down the mid-line. Material was fixed in 10 p.c. formol-saline. Brain and muscle suspensions containing active virus were prepared by grinding up the tissue with sterile washed sand in a pestle and mortar to give a 10 p.c., 20 p.c. or 30 p.c. suspension. The diluting fluid for the myositis-producing strains was neo-peptone-heart broth. Saline containing 10 p.c. rabbit serum was used as a diluent for encephalitis-producing strains. The debris was allowed to settle and the supernatant removed and frozen after the addition of penicillin and streptomycin in concentrations of 100 units/ml. and 0·5 mg./ml. respectively.

EXPERIMENTAL.

Susceptibility to infection of mice of various ages.

Virus material from the first, second, third or fourth passages was inoculated by the intracerebral or intraperitoneal route into 24 hour old litters, 5 day old litters and also into 21 day old and adult mice. The results are recorded in Table 1.

TABLE 1.

Age susceptibility of mice to suckling-mouse viruses.

Virus	Inoculum	Age of mice							
		1 day		5 days		21 days		Adults	
		IC	IP	IC	IP	IC	IP	IC	IP
W32	30 p.c. limb suspension	—	3	3–4	3	S	S	S	S
J33	30 p.c. limb suspension	—	3	3	3	S	S	S	S
J37	30 p.c. limb suspension	—	2–3	2–3	2–3	S	S	S	S
Sewage 4	30 p.c. limb suspension	—	2–3	3	2–3	S	S	S	S
E46	20 p.c. limb suspension	—	2–3	3–4	3–4	S	S	S	S
P29	20 p.c. brain suspension	—	4–5	6–8	6–8	5–9	6–9	6–12	10–14*
B48	20 p.c. limb suspension	—	2–4	3–5	6–8	3–5	6–9	5–10	9–16*

Numbers = days to produce paralysis and/or death. IC = intracerebral.
S = all animals survived. IP = intraperitoneal.
— = test not performed.
* Mice not as susceptible to infection by IP route as by IC route. (Approximately 10 p.c. fatalities.)

It may be seen that the strains can be divided into two groups—one group capable of paralysing and killing *only* suckling mice a few days old, and the other affecting 21-day-old and adult mice as well as suckling mice.

Signs of infection in suckling mice.

Infection with the myositis producing viruses resulted in a picture that showed little variation. The consistent features were retardation of growth, paralysis of limbs with occasional wrist drop and a moribund condition several hours prior to death. Infection with the encephalitis producing strains (P29, B48 and E46) produced different syndromes which appeared to be as much a function of the incubation time as the type of virus. The most constant features observed were retardation of growth followed by paralysis, after which the animals became moribund and died. A blood-stained peritoneal exudate was recorded only with P29 and B48 (herpes strains). Tremors were often a marked feature of infections with B48 and E46 viruses, while cyanosis was occasionally noted in mice infected with E46 and P29.

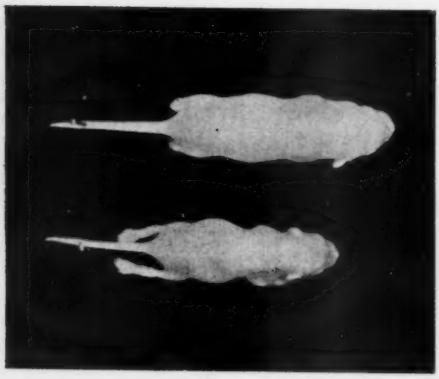

Fig. 1.

Fig. 2.

Fig. 1. P29 virus (herpes simplex). Lower mouse: inoculated by the intraperitoneal route 5 days prior to death (Passage IV). Upper mouse: non-infected litter mate of same age.

Fig. 2. E46 virus (Dalldorf Group B). Lower mouse: inoculated by the intraperitoneal route 11 days prior to death (Passage IV). Upper mouse: non-infected litter mate of same age.

Although retardation of growth was obvious in mice dying between the second and fourth days, the effect was more marked if the time of death was delayed by the inoculation of smaller doses of virus. This was most noticeable in the titrations of brain and muscle suspensions. Fig. 1 shows the difference between infected and non-infected litter mates killed five days after inoculation, and Fig. 2 shows the difference between infected and non-infected litter mates killed eleven days after inoculation.

Histological findings.

At least five passages were performed with each virus, and material from dying animals was examined histologically. Pathological changes could not be detected in the liver, spleen, kidney, suprarenal or pancreas of any animal. Destruction of the pancreatic acini, which

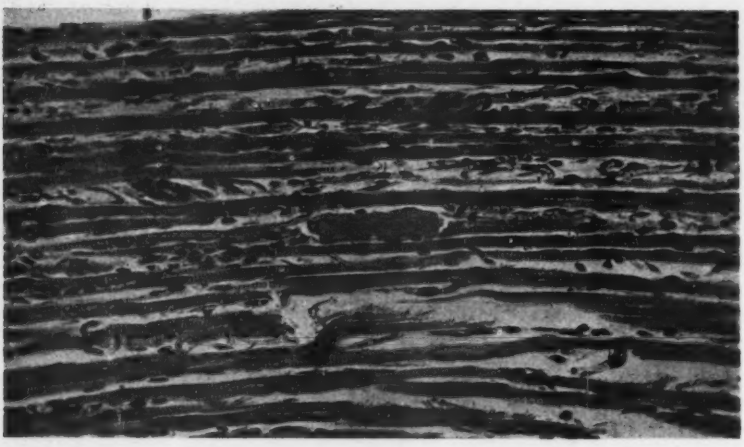

Fig. 3.

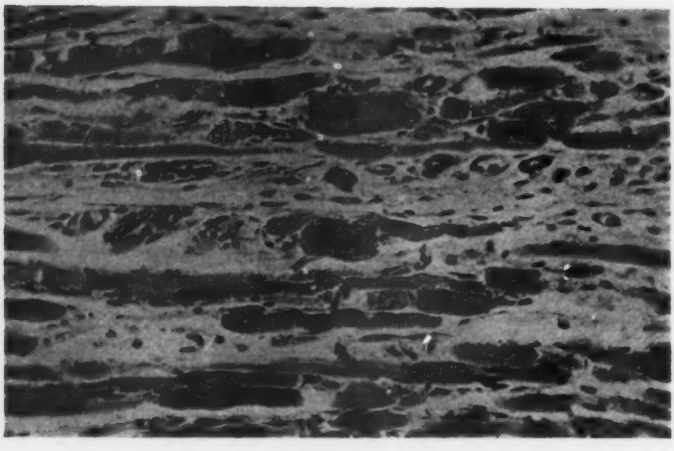

Fig. 4.

Fig. 3. Section of leg muscle of suckling mouse paralysed as a result of infection with a myositis-producing strain (Sewage 4). Haematoxylin-eosin stain. Magnification 500×.

Note one acidophilic swelling with loss of striation compared with apparently unaffected muscle fibres.

Fig. 4. The same slide and magnification as in Fig. 3. Note extensive destruction of skeletal muscle with few normal muscle fibres remaining.

is characteristic of infection with the Powers virus (Pappenheimer, Daniels, Cheever and Weller, 1950), was not observed. Strains W32, J33, J37 and Sewage 4 gave an identical picture in that they all produced myositis of the skeletal muscle without evidence of pathological change elsewhere. The changes consisted of acidophilic swellings of the muscle fibres, followed by disintegration and phagocytosis (see Figs. 3 and 4). Although no changes were observed in the central nervous systems of mice dying from infection with these four strains, the virus could be demonstrated therein (see Table 4). Of the remaining three strains, P29 and B48 produced lesions in the central nervous system without affecting the skeletal muscle. Strain E46 produced mainly lesions in the central nervous system, while changes of the skeletal muscle, although present to a mild degree in some sections, were rarely seen. Infection with P29 virus resulted in perivascular infiltration and a diffuse type of encephalitis with pyknosis and karyorrhexis. Focal necrosis and concentrations of glial cells were obvious in some preparations. A marked meningitis was seen in some sections prepared from adult mice—a feature not yet detected in suckling mice. Densely staining acidophilic bodies thought to be intranuclear inclusions were most marked with this virus, but were only occasionally demonstrable with B48 virus. Infection with the latter virus resulted in focal necrosis, concentrations of glial cells and perivascular infiltration.

These observations show that on histological grounds the viruses may be divided into two groups: four myositis-producing types and three encephalitis types. The two viruses pathogenic for adult mice differ from the others in producing bodies thought to resemble the inclusions characteristic of herpetic infection.

Resistance of viruses to heat and germicides.

Tissue suspensions (20 p.c.) prepared in the usual way from infected animals were used. The results recorded in Table 2 show that some strains survived a temperature of 55° C. for 30 minutes, while strains J37 and Sewage 4 were not completely inactivated until they had been heated for 30 minutes at 65° C.

TABLE 2.

Heat susceptibility of suckling-mouse viruses.

Virus strain		Unheated control	Temperature			
			55° C.		65 ° C.	
			10 mins.	30 mins.	10 mins.	30 mins.
Myositis producing strains	W32	8/8	0/8	0/9	—	—
	J33	9/9	0/10	0/11	—	—
	J37	8/8	8/8	8/8	2/7	0/10
	Sewage 4	8/8	—	7/7	0/8	0/9
Encephalitis producing strains	E46	8/8	3/6	3/7	0/8	0/8
	P29*	8/8	0/7	0/5	—	—
	B48*	7/7	2/9	0/8	—	—

Numerator = number of suckling-mice observed with paralysis and/or death.
Denominator = number of suckling-mice in the litter inoculated.
— = test not performed.

* These strains are also pathogenic for adult mice (see text).

The viruses appeared not to be affected by diethyl-ether, penicillin, streptomycin or aureomycin. Germicidal tests with W32 strain revealed this virus to be resistant to 70 p.c. ethanol, 2 p.c. lysol and 1/10,000 merthiolate, and inactivated by 0·25 p.c. formalin (see

Table 3). In each case virus material was recovered from the mixture of germicide plus virus by diluting and then concentrating in the ultracentrifuge prior to inoculation of mice.

TABLE 3.

Susceptibility of W32 strain to germicides.

Germicide	Time of contact	
	1 hour	24 hours
2 p.c. lysol	10/10	7/7
0·25 p.c. formalin	0/5	—
1/10,000 merthiolate	5/5	7/7
70 p.c. ethanol	4/4	6/6

Numerator = number of suckling mice paralysed or dead.
Denominator = number of suckling mice in litter inoculated.

Concentrations of virus in brain and muscle.

These were determined by making ten-fold dilutions of freshly harvested infected tissue in broth or serum saline. Each dilution was inoculated by the intraperitoneal route into one litter (average 8 mice). The results are recorded in Table 4. The infective dose 50 (ID_{50}) was determined by the method of Reed and Muench (1938).

TABLE 4.

Infective dose 50 (ID_{50}) for suckling-mouse viruses.

Virus	ID_{50} of 10 p.c. suspensions of	
	Limbs	Brains
J33	10^{-6}	10^{-3}
W32	10^{-7}	10^{-4}
J37	10^{-6}	10^{-5}
Sewage 4	10^{-6}	10^{-4}
E46	10^{-5}	10^{-6}
P29	10^{-2}	10^{-3}
B48	10^{-1}	10^{-2}

Tests for neutralizing antibodies.

The concentration of virus employed in each test was approximately 100 ID_{50}. The serum to be tested for neutralizing antibodies was diluted in broth or serum saline, and equal volumes of virus suspension and serum dilutions were allowed to stand at room temperature for 1 hour. Each dilution was inoculated into a litter by the intraperitoneal route. The virus controls were allowed to stand for 1 hour in normal mouse serum at room temperature before inoculation. The results are recorded in Table 5.

It may be seen from these Tables that the myositis-producing strains fall into two distinct types; W32 and J33 appear to be serologically identical and unrelated to the Sewage 4 and J37 types.

TABLE 5.

Neutralization tests.

Virus strain	Control Normal mouse serum 1/20	Mouse serum dilutions							
		W32				J33			
		1/20	1/80	1/320	1/1,280	1/20	1/80	1/320	1/1,280
W32	39/40	0/9	0/8	0/10	0/8	0/8	0/7	0/5	0/8
J33	20/20	0/7	0/6	0/8	0/8	0/6	0/8	0/8	0/8
Sewage 4	16/16	8/8	8/8	8/8					
J37	17/17	7/7	9/9	8/8	7/7				
E46	28/28	9/9	4/4	6/6					

TABLE 5 (Continued).

Neutralization tests.

Virus strain	Control Normal mouse serum 1/20	Mouse serum dilutions											
		Sewage 4				J37				P29			
		1/20	1/80	1/320	1/1,280	1/20	1/80	1/320	1/1,280	1/20	1/80	1/320	1/1,280
W32	39/40	9/9	6/6	6/6	9/9	8/8	7/7	8/8	5/5	6/6	6/6	8/8	
J33	20/20												
Sewage 4	16/16		0/9	0/7	1/6	0/7	0/8	0/8	4/8				
J37	17/17					0/7	0/8	0/9	0/8				
E46	28/28	2/8	3/7	9/9	6/6					8/8	9/9	7/7	

Numerator = number of mice paralysed and dying.
Denominator = number of mice in litter(s) inoculated.

Neutralization tests with the two strains (P29 and B48) pathogenic for adult mice as well as for suckling mice were carried out by a similar technique. Immune mouse serum prepared against these strains was tested against known strains of herpes simplex. One of these was the HFE strain[1] which had been passaged many times on the chorioallantois of chick embryos and the other was a freshly isolated strain (K98) obtained by inoculation of suckling mice with fluid from vesicles. The results of these tests shown in Table 6 are recorded differently from the other neutralization tests to show the effect of specific antibody on the time taken to produce signs of infection or death.

It can be seen that, although both B48 and P29 antiserum neutralized both strains of herpes simplex, the P29 serum possessed a higher titre.

Further tests identifying P29 and B48 viruses as strains of herpes simplex.

Both strains were adapted to grow on the chorioallantoic membrane of 13-day-old chick-embryos and produced pocks characteristic of herpes simplex. B48 virus was readily adapted, but P29 only with difficulty. It was found during these experiments and also those with

[1] Obtained through the courtesy of Mr. E. L. French, of the Walter and Eliza Hall Institute, Melbourne.

suckling mice that the P29 strain was much more labile and more easily inactivated at room temperature than the B48, K98 or HFE herpes strains.

Corneal scarification of rabbits was readily followed by a keratitis leading finally to a fatal encephalitis in the case of B48 virus. A keratitis was produced once only with P29 virus. On the other hand, intranuclear inclusion bodies characteristic of herpes infection of the central nervous system were quite common with P29 virus, but uncommon with the B48 strain.

TABLE 6.

Suckling-mouse neutralization tests with P29 and B48 antisera against known strains of herpes simplex.

Strains of herpes virus	Serum	No. of days to produce paralysis and/or death													No. of S.M. in litter	No. of S.M. dying
		1	2	3	4	5	6	7	8	9	10	11	12	13		
K98 (1 hour neutralization time)	Normal	—	—	—	6*										6	6
	P29 1/20	—	—	—	—	—	—	—	—	—	—	—	—	—	7	0
	P29 1/40	—	—	—	—	—	—	1	—	—	—	—	2	—	6	3
	P29 1/80	—	—	—	—	—	—	—	—	—	1	1	—	—	7	2
	P29 1/160	—	—	—	—	—	6								6	6
	B48 1/20	—	—	—	—	—	—	—	1	1	1	1	—	—	7	4
	B48 1/40	—	—	—	—	—	—	—	2	—	1	—	—	—	4	3
	B48 1/80	—	—	—	—	—	6	1							7	7
	B48 1/160	—	—	—	—	—	5	—	—	1					6	6
HFE (3 hours neutralization)	Normal	—	—	—	2	2	2								6	6
	P29 1/20	—	—	—	—	—	—	—	—	—	—	—	—	—	8	0
	P29 1/40	—	—	—	—	—	—	—	—	—	—	—	—	—	7	0
	P29 1/80	—	—	—	—	—	—	—	—	—	—	—	—	—	6	0
	P29 1/160	—	—	—	—	—	—	—	—	—	—	—	—	—	8	0
	B48 1/20	—	—	—	—	—	—	—	—	—	—	—	—	—	6	0
	B48 1/40	—	—	—	—	—	—	—	—	—	—	—	—	—	10	0
	B48 1/80	—	—	—	—	—	—	—	—	—	—	—	—	—	7	0
	B48 1/160	—	—	—	—	—	—	—	—	—	—	—	—	—	9	0

* Numbers refer to mice paralysed or dead.

Antisera prepared against B48 and P29 viruses neutralized HFE and K98 herpes strains when tested by inoculation of the chorioallantoic membrane of the developing chick embryo with serum-virus mixtures.

DISCUSSION.

It is likely that the ultracentrifugation of throat washings and faecal material increases considerably the chances of isolating viruses. Tests with infected muscle suspensions have shown that more than 99 p.c. of the virus is sedimented at 40,000 r.p.m. (144,000 *g*) for 1 hour at 4° C. Furthermore, when concentrated material from throat washings or faeces was primarily inoculated, in many cases only one or two mice per litter showed evidence of infection. Penicillin and streptomycin, in preference to filtration procedures, were used to kill the few bacteria remaining after centrifugation. Although infective material is filtrable through such filters as Seitz E.K., some inactiva-

tion is thought to occur. With the technique described, six of twenty patients (30 p.c.) were shown to carry in the throat washings or stools viruses capable of causing myositis or encephalitis in suckling mice. In some cases, the virus isolated was thought to be the causative agent of the infection as indicated by the estimation of neutralizing antibodies in the patient's serum collected during the acute and convalescent stages. This aspect of the work will not be discussed here. At this stage, however, it does seem more practical to identify cases by isolation of viruses rather than by serological tests on the patients' serum.

The virus strains isolated showed interesting differences. The E46 strain gives every indication that it is similar to Dalldorf's group B Coxsackie viruses in that it produces mainly encephalitis in infant mice. The remaining strains, W32, J33, J37 and Sewage 4 appear to be identical with the myositis-producing type of Coxsackie virus designated by Dalldorf as group A (Dalldorf, 1950a). Neutralization tests with sera prepared against these strains showed that they fell into two types, W32 and J33 composing one, and J37 and Sewage 4 the other. There appears to be some antigenic overlap between E46 encephalitis type and J37 myositis types (see Table 5). A correlation between serological properties and other features such as susceptibility to heat, time of onset and the syndrome characteristic of infection, was noted. As a rule, when 10 p.c. suspensions were inoculated, the true suckling mouse viruses had an incubation period of about 48 hours. Considerable variation was noticed with the herpes strains, but the incubation period was generally much longer. The titration figures showed, as was to be expected, that the time of death or of onset of symptoms was dependent on the dose of virus injected. For example, with W32 strain, all mice in litters inoculated with concentrations of 10^{-2} or higher became paralysed or died within two to three days. With concentrations of 10^{-5} to 10^{-7}, the suckling mice may take as long as seven to ten days to develop symptoms. Generally speaking, one could predict to the day the time of onset for each virus, if the dose were known. Such properties did not appear to be altered by many passages in suckling mice. The concentration of virus in the brains of suckling mice paralysed and dying from the disease was not as great as that in muscle, although infected brains had an ID_{50} of between 10^{-3} and 10^{-5}. There were, however, no apparent histological changes observed in the central nervous system as a result of the virus concentration therein. This observation is in accord with those made by Dalldorf (1950) and Melnick, Clarke and Kraft (1950).

In our hands, the neutralization test appeared to be more successful than the complement-fixation test for detection of antibodies It is likely that the preparation of a suitable complement-fixation antigen presents some difficulties in this regard (Kraft and Melnick, 1950). Most of the mouse antisera pre-

pared against the myositis-producing strains contained neutralizing antibodies to high titre, e.g. >1/10,000. With the encephalitis-producing strain (E46) and the herpes strains lower titres were obtained for neutralizing antibodies.

The sensitivity of the strains to heat and germicides was studied. There appears to be some variation in heat sensitivity that could be correlated with the serological type. The W32 and J33 strains were inactivated much more rapidly than strains belonging to a different serological type. The tests done with W32 virus indicate that it is resistant to the actions of penicillin, streptomycin, aureomycin, 2 p.c. lysol, merthiolate and 70 p.c. ethanol for at least 24 hours. Contact with 0·25 p.c. formalin for 1 hour, on the other hand, completely inactivated a concentrated muscle suspension of the virus. Formalin was therefore used in preference to lysol to swab down benches after experiments.

It is unlikely that the viruses isolated were latent viruses recovered from the mouse stock. Before these experiments were commenced numerous samples of suckling mouse tissue (brain, muscle) were ground up in broth and inoculated by the intracerebral and intraperitoneal routes into litters of mice less than 48 hours old. There was no evidence of infection. Furthermore, in the course of the experiments outlined in this communication, more than five thousand mice were inoculated without giving any evidence of the presence of a latent virus.

The Coxsackie group of viruses has come to include a number of viruses, infection with which results in pathological changes in many different organs (Melnick and Godman, 1951). The one common feature possessed by the group appears to be their pathogenicity for suckling mice and hamsters, but not for adult animals of these species. Although members of this group may give rise to diverse syndromes in humans, there is evidence (Weller, Enders, Buckingham and Finn, 1950) of immunological relationships between certain strains, e.g. the DeMole virus isolated from a case of pleurodynia (Weller *et al., loc. cit.*) and the Connecticut 5 virus isolated from a case of "non-paralytic poliomyelitis" (Melnick, Shaw and Curnen, 1949). Our investigations have shown that infant mice may be used to isolate viruses which are quite distinct from those originally designated by Dalldorf as "Coxsackie" viruses, and which, although pathogenic for adult mice and rats, produce in suckling mice an encephalitis similar to that resulting from infection with some viruses of the Coxsackie group. Myositis has not been observed with the two encephalitis strains, (P29 and B48) which give reactions characteristic of herpes simplex. Mice paralyzed as a result of infection with either B48 or P29 herpes strains are often impossible to distinguish from suckling mice infected with Coxsackie viruses. The blood-stained peritoneal exudate characteristic of herpetic infection of suckling mice was shown to contain herpes virus in approximately the same concentration as that found in the central

nervous system of infected mice. The importance of determining the age susceptibility of mice to viruses primarily isolated in suckling mice is obvious. Our work indicates that Coxsackie viruses are isolated more readily from faeces than from throat washings. Although we failed to isolate Coxsackie viruses from throat washings, the isolation of herpes strains therefrom is in keeping with the finding of Dalldorf (1950b).

TABLE 7.

Summary of properties of Australian viruses.

	Virus strain	Isolated from	Pathogenic for		Histological lesions		Inactivated by heat at 55° C. for 30 mins.
			Suckling mice	Adult mice	Myositis	Encephalitis	
Coxsackie strains	J33	faeces	+	—	+	—	+
	W32	faeces	+	—	+	—	+
	Sewage 4	faeces	+	—	+	—	—
	J37	faeces	+	—	+	—	—
	E46	faeces	+	—	rare	+	+
Herpes strains	P29	throat washings	+	+	—	+	+
	B48	throat washings	+	+	—	+	+

	Virus strain	Serological type	Probable Dalldorf group	ID_{50}		Encephalitis in rabbits		Pocks on chorioallantois
				Brain	Muscle	I.V. inoculation	Corneal scarification	
Coxsackie strains		W32	A	10^{-3}	10^{-6}			—
		W32	A	10^{-4}	10^{-7}			
	Sewage 4	J37	A	10^{-5}	10^{-7}			
	J37	J37	A	10^{-5}	10^{-6}			—
	E46	—	B	10^{-6}	10^{-5}			—
Herpes strains	P29			10^{-3}	10^{-2}	—	+	+
	B48			10^{-2}	10^{-1}	+	+	+

It is not the purpose of this paper to discuss the aetiology of paralytic poliomyelitis and non-paralytic poliomyelitis, nor the significance of the suckling mouse viruses in these conditions. Until our tests for the presence of poliomyelitis viruses by monkey inoculations have been completed such dis-

cussion would be of little value. It is felt, however, that this work demonstrates that, with a suitable technique, the suckling mouse can be used to isolate viruses (Coxsackie and herpes) from patients whose condition clinically resembles acute anterior poliomyelitis. Since the experimental work in this paper was completed, suckling mice have been used for the primary isolation of other strains of herpes, thus indicating the suitability of this host for the purpose. In this laboratory, fatal infections of suckling mice have been demonstrated by intracerebral inoculation with the MEF strain of poliomyelitis as well as Theiler's encephalomyelitis virus. All neutralization tests, titrations and primary isolations with Coxsackie viruses were carried out by intraperitoneal inoculation.

The ease with which one myositis-producing strain was recovered from raw sewage suggests that such viruses were widespread in Sydney at the time these investigations were being carried out, that is, at the time of maximum incidence of the 1950–51 epidemic of poliomyelitis.

Some of the properties of the seven strains studied here have been summarized in table form (Table 7).

SUMMARY.

The isolation and properties of seven viruses pathogenic for suckling mice are described. The strains were recovered from the faeces or throat washings of patients admitted to hospital with a provisional diagnosis of "poliomyelitis." Evidence has been produced which indicates that those viruses isolated from faeces are similar to those of the Coxsackie group recently isolated in the U.S.A., while those strains isolated from throat washings were shown to be strains of herpes simplex. The faecal strains consisted of three serological types. Four of the faecal strains produced myositis only, while infection with the remaining strain produced mainly encephalitis. The suitability of suckling mice for the isolation of viruses other than those of the Coxsackie group has been demonstrated.

REFERENCES.

Armstrong, M. P., Wilson, F. H., McLean, W. J., Silverthorne, N., Clark, E. M., Rhodes, A. J., Knowles, D. S., Ritchie, R. C. and Donohue, W. L. (1950): Canad. J. pub. Health, 41, p. 51.

Casals, J., Olitsky, P. K. and Murphy, L. C. (1949): Proc. Soc. exp. Biol., 72, p. 636.

Cheever, F. S., Daniels, J. B. and Hersey, E. F. (1950): J. exp. Med., 92, p. 153.

Curnen, E. C. (1950): Bull. N.Y. Acad. Med., 26, p. 335.

Curnen, E. C., Shaw, E. W. and Melnick, J. L. (1949): J. Amer. med. Assoc., 141, p. 894.

Dalldorf, G. (1949): Science, 110, p. 594.

Dalldorf, G. (1950a): Bull. N.Y. Acad. Med., 26, p. 329.

Dalldorf, G. (1950b): Amer. J. pub. Health, 40, p. 1,508.

Dalldorf, G. and Sickles, G. M. (1948): Science, 108, p. 61.

Dalldorf, G., Sickles, G. M. and Plager, H. (1949): J. exp. Med., 89, p. 567.

Findlay, G. M. and Howard, E. M. (1950): Brit. med. J., i, p. 1,233.

Howitt, B. F. (1950): Proc. Soc. exp. Biol., 73, p. 443.

Howitt, B. F. and Benefield, U. R. (1950): *Ibid.*, 73, p. 90.

Huebner, R. J., Armstrong, C., Beeman, E. A. and Cole, R. M. (1950): J. Amer. med. Assoc., 144, p. 609.

Huebner, R. J., Cole, R. M., Beeman, E. A., Bell, J. A., Peers, J. H. (1951): *Ibid.*, 145, p. 628.

Kraft, L. M. and Melnick, J. L. (1950): J. exp. Med., 92, p. 483.

Manire, G. P., Sulkin, S. E. and Farmer, T. W. (1950): Proc. Soc. exp. Biol., 73, p. 341.

Melnick, J. L. and Godman, G. C. (1951): J. exp. Med., 93, p. 247.

Melnick, J. L. and Kaplan, A. S. (1950): Proc. Soc. exp. Biol., 74, p. 812.

Melnick, J. L. and Ledinko, N. (1950): J. exp. Med., 92, p. 463.

Melnick, J. L., Clarke, N. A. and Kraft, L. M. (1950): *Ibid.*, 92, p. 499.

Melnick, J. L., Shaw, E. W. and Curnen, E. C. (1949): Proc. Soc. exp. Biol., 71, p. 344.

Pappenheimer, A. M., Daniels, J. B., Cheever, F. S. and Weller, T. H. (1950): J. exp. Med., 92, p. 169.

Reed, L. J. and Muench, H. (1938): Amer. J. Hyg., 27, p. 493.

Sickles, G. M. and Dalldorf, G. (1949): Proc. Soc. exp. Biol., 72, p. 30.

Stanley, N. F. and Hayes, L. (1950): Austral. J. Sci., 13, p. 81.

Weller, T. H., Enders, J. F., Buckingham, M. and Finn, J. J. (1950): J. Immunol., 65, p. 337.

THROMBIN COMPONENTS IN BLOOD COAGULATION

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It has been regarded as axiomatic in present day theories of blood coagulation that the action of thrombin upon fibrinogen is a simple one, and that thrombin itself is a unit substance. Lyons (1945a, b) and Laki and Mommaerts (1945) suggested a two-stage process in the clotting of fibrinogen. Lyons produced evidence that fibrinogen went through a well-defined intermediate stage which he called fibrinogen B, before it was finally converted to fibrin. Lyons *et al.* (1948),(1949) also showed that this intermediate stage, fibrinogen B, could be demonstrated in certain pathological plasmas. In healthy normal subjects the incidence of fibrinogen B is very low. One hundred and five football players were examined both before and after play. Of these two showed a positive fibrinogen B reaction before play and the same players and one other previously negative gave a positive reaction after play.

Since the two forms of fibrinogen were so sharply defined, five possibilities were considered as to their significance in the thrombin fibrinogen reaction.

1. That the formation of fibrinogen B is not connected with the action of thrombin.
2. That the transformation of fibrinogen to fibrin by thrombin could be inhibited at the stage of fibrinogen B by an anti-thrombin.
3. That the thrombin molecule contains two active groups.
4. That thrombin could be composed of at least two components, thrombin A and thrombin B.
5. That thrombin A and thrombin B are by-products of the thrombin fibrinogen reaction.

Examing these possibilities in the order cited:

Number 1 was untenable, as a fibrinogen B is always formed as an intermediate stage in the normal conversion of fibrinogen to fibrin. Whenever the fibrinogen B test is performed after one half of the known clotting time has elapsed, a maximal amount of fibrinogen B is demonstrated. As no fibrinogen can be detected after the removal of the gel formed by the test, it appears that all the fibrinogen had been transformed to fibrinogen B.

[1] Work done under a grant from the National Health and Medical Research Council.

Number 2 would be tenable if large quantities of heparin or any anti-thrombin could be demonstrated in the circulating blood. The naturally occurring anti-thrombin activity in blood does not seem sufficient to lend probability to this hypothesis. However, the introduction of small amounts of heparin into a thrombin-plasma mixture shows that there is little inhibition of the formation of fibrinogen B even when clotting is totally inhibited.

Number 3. This hypothesis is acceptable, and in our experience equally tenable with Number 4. It appears possible that upon reacting with fibrinogen, thrombin is split into two components, both of which have been isolated.

Number 4. This hypothesis is the most acceptable because both components can be demonstrated separately, and clotting of fibrinogen will not occur unless both are present. There is strong indirect evidence which suggests that thrombin could be a unit (thrombin AB) which upon reacting with fibrinogen in normal blood is converted to thrombin A and thrombin B, and that after clotting has occurred the thrombin A and thrombin B may re-combine partially to form thrombin AB. Preliminary electrophoretic studies in this laboratory indicate at least two components of thrombin.

Number 5. This is a modification of Number 4, and has two obvious disadvantages. (a) The action of thrombin is generally considered to be enzymatic. (b) Thrombin A must act on fibrinogen before any clot is formed. Thrombin A and thrombin B may be end-products as well as initiators of clotting, due to the enzymatic function of thrombin.

The components of thrombin are discussed in the order in which they act according to the clotting mechanism:

$$\text{Fibrinogen A} \xrightarrow{\text{Thrombin A}} \text{Fibrinogen B} \xrightarrow{\text{Thrombin B}} \text{Fibrin.}$$

Materials.

Thrombin solution. The thrombin used throughout these experiments, except where otherwise stated, was Thrombin Topical (Parke Davis) of bovine origin. As a diluent, sterile physiological saline was used in place of the ampoule of saline supplied, which contained a preservative, "Phemerol."

Prothrombin was prepared by the method of Quick and Stefanini (1949) and is called component A by Quick. As it resembles the classical concept of prothrombin it is referred to throughout this paper by the name "prothrombin."

Silicone. The silicone referred to throughout this paper is Dri-film 9987, manufactured by the General Electric Corporation, Schenectady, N.Y.

Platelets were prepared by differential centrifuging, and lysis was completed by using 1:100,000 saponin in saline. Platelets from 50 ml. of blood were resuspended in final concentration of 5 ml. of the saponin saline solution.

Thromboplastin was prepared from rabbit brain according to the method of Quick (1942).

Calcium phosphate plasma was prepared by adsorbing plasma with tricalcium phosphate as described by Quick (1942).

Lyons' reagent was prepared by dissolving 2 gm. of beta-naphthol in 100 ml. of 50 p.c. ethyl alcohol and passing a current of air through slowly for twelve hours.

pH. A Cambridge pH meter was used in the determination of pH and unless otherwise stated all solutions were brought within the range pH 7·1–7·5.

Anticoagulants.

Oxalate solution was made up by dissolving 1·34 gm. of sodium oxalate in distilled water neutralizing to pH 7·3 and making up to a total volume of 100 ml. One volume of this solution was used to 9 volumes of blood.

Citrate solution. 3·8 gm. of sodium citrate were dissolved in distilled water neutralized, and the volume was made up to 100 ml. Two volumes of this solution were mixed with 8 volumes of blood.

Naphthoquinone reagent was prepared according to the method of Lyons (1945a).

Blood samples: technique and materials.

Syringes used in these experiments were the Yale BD-Lok Syringe type and the needles used were No. 19. Unless otherwise stated both syringes and needles were coated with silicone according to the method of Jaques *et al.* (1946). In performing the venepuncture, the vein was entered immediately by intention, with one clean stab. If this was not achieved, the blood sample was discarded. Excess eddy currents and particularly frothing were avoided in the obtaining of the blood sample.

EXPERIMENTAL.

Test for fibrinogen B in plasma.

In our experience of over 10,000 fibrinogen B tests it became apparent that the optimal amount of reagent is not always constant, and that a definite volume to volume ratio cannot always be stipulated. When the reagent is added in excess the fibrinogen is precipitated immediately, before a gel can form. If too little reagent is added, either the reagent is inactivated by other proteins of the plasma, or the rate of reaction is too slow for the gel to appear in the ten minutes stipulated for the test. For this reason, a slight modification is used in the method which was described previously by Cummine and Lyons (1948) and three tubes are used in place of one. To the first tube, Lyons' reagent is added, drop by drop, until slight opalescence appears (N drops required). To tube No. 2 (N–1 drops) are added. To the third tube (N + 1) drops are added. If the substance profibrin is present in the blood sample an immediate gel forms in all three tubes upon adding the reagent, (this gel will also form immediately upon the addition of sufficient 50 p.c. alcohol). Usually no gel forms immediately upon the addition of the reagent and the tubes are shaken, then allowed to stand undisturbed for ten minutes when they are examined for gel formation. The appearance of a frail gel is designated as fibrinogen B+, the appearance of a stronger gel designated as fibrinogen B++, and a gel which keeps the contour of the end of the tube upon tilting is designated as fibrinogen B+++. The tests were all performed at room temperature (16° to 21° C.).

Thrombin A.

During experimental work in the laboratory it was found that a number of pseudo-positive reactions were occurring when silicone coated syringes were being used but not when an ordinary glass syringe was used. These pseudo-positive reactions were particularly evident

after a solution of thrombin plus a very dilute solution of fibrinogen had been aspirated into the syringe. These phenomena also occur when siliconed syringes are used repeatedly for obtaining blood samples without meticulous cleansing by boiling. It would seem that continual exposure to blood samples undergoing the preliminary changes associated with clotting was sufficient to cause these phenomena. It was found that the silicone had adsorbed a substance which could be eluted by sodium citrate solution, and that this could convert normal fibrinogen to fibrinogen B without the change proceeding any further to fibrin. This adsorbed and eluted substance had all the properties attributed to thrombin A by Lyons (1945b). Once adsorbed upon silicone, thrombin A remains active for days, and will convert the fibrinogen of any blood which contacts that surface to fibrinogen B+++.

Although sodium citrate will elute the adsorbed thrombin A, repeated washing with distilled water or phosphate buffer has no effect. Whilst it might appear that the silicone surface has an orientating effect upon the fibrinogen molecule, this appears improbable as the active substance can be eluted by citrate. Further confirmation is supplied by Horan *et al.* (1950), who found that the z-potential of silicone is close to that of glass at pH 6·9.

Preparation of thrombin A.

Two experiments demonstrating the adsorption of thrombin A upon silicone are cited here as examples.

Experiment No. 1. Five ml. of bovine thrombin were aspirated into a freshly siliconed syringe and allowed to stand for one hour. The thrombin was then removed and the syringe thoroughly washed with phosphate buffer and distilled water. Five ml. of normal plasma were then aspirated into the syringe and allowed to stand for 30 minutes. The plasma was then tested and found to be fibrinogen B+++. The syringe was thoroughly washed after removal of the plasma and 0·2 M. sodium citrate was aspirated into the syringe and allowed to stand for one hour. This eluate was then tested for thrombin A activity on normal plasma (which contained no fibrinogen B). Mixtures of normal plasma and the washings obtained from the syringe were tested with Lyons' reagent after standing 15 minutes, and the production of fibrinogen B was demonstrated. (Table 1).

TABLE 1.

Elution of thrombin A from silicone.

Normal plasma	Phosphate buffer washings	Distilled water washings	Citrate eluate	Fibrinogen B test
0·5 ml.	0·5 ml.	—	—	—ve
0·5 ml.	—	0·5 ml.	—	—ve
0·5 ml.	—	—	0·5 ml.	+++

Experiment No. 2. Blood obtained by venepuncture was allowed to stand in a siliconed syringe for 15 minutes, at the end of which time no clot had formed. The plunger was withdrawn and both barrel and plunger were thoroughly washed with distilled water. Four ml. of 0·2 M. sodium citrate were aspirated into the syringe and shaken for a few minutes. The eluate was then added to normal plasma and fibrinogen B was rapidly formed. The sodium citrate solution which was used for elution, had no effect *per se*.

Thrombin B.

Thrombin B is characterized by its ability to clot fibrinogen B positive plasmas whilst it will not affect normal plasma. It is rapidly inactivated by plasma proteins, especially in the presence of heparin; and is not readily adsorbed by tricalcium phosphate. Its action can be simulated by various organic chemicals, Chargaff and Ziff (1941); Chargaff and Bendich (1943); Lyons (1945b); Dunn, Jackson and Lyons (1949); Jenner (1947).

Preparation of thrombin B.

In order to standardize procedure, Thrombin Topical (Parke Davis) was used. 5,000 units of the dried protein were dissolved in 100 ml. of distilled water and adsorbed with an equal volume of 0·008 M. tricalcium phosphate, in the manner used by Quick (1942), for his prothrombin adsorptions. The supernatant of this adsorption showed no thrombin activity. Plasmas containing fibrinogen B were clotted (see Table 2) but not plasmas from normal healthy subjects irrespective of whether the anticoagulant was oxalate or citrate.

TABLE 2.

Thrombin B in human blood coagulation.

Substrate	Anticoagulant	Fibrinogen B test	Thrombin B*
Normal plasma (pH 7·3)	Sodium oxalate	Negative	No clot 30 mins.
Pathogenic plasma (pH 7·3)	Sodium oxalate	+++	Clot 17 secs.
(0·5 ml.) Normal plasma + 0·5 ml. citrate eluate**	Sodium oxalate	+++	Clot 11 secs.

* Supernatant of tricalcium phosphate adsorption (pH 7·3). See text.

** Same eluate as described for Table 1.

Origin of thrombin A and thrombin B.

Efforts to combat hypercoagulability in human blood have mostly been concentrated upon the prothrombin ——→ thrombin conversion in an effort to inhibit this first stage. Yet it is the fibrinogen ——→ fibrin transformation which is ultimately concerned with the formation of a clot. The importance, both theoretical and practical of a two-stage clotting mechanism in the action of thrombin upon fibrinogen, stimulated research into the above factors, which I believe influence the formation and clotting of fibrinogen B. It seems logical that the above originate from the prothrombin-complex.

An accessory factor of prothrombin has been named by various investigators, labile factor, Quick (1943), prothrombin accelerator, Fantl and Nance (1946), factor V, Owren (1947), accelerator globulin, Ware, Murphy and Seegers (1947), but in this investigation is called accessory factor because of this multiplicity of names. It seems very probable that all these substances are one and the same. Since Quick (1943) first suggested in 1941 that there were at least two prothrombins and subsequent work of Oneal and Lam (1945) Munro

et al. (1945), Owren (1947), Feissly, (1945), Fantl and Nance (1946), and Ware and others (1947) has confirmed this finding, it was of interest to investigate whether the two components of thrombin A and B had separate precursors. In our investigation to date, it appears that both components of thrombin derive from the prothrombin A of Quick (1949). Zondek and Finkelstein (1945), and later Honorato (1947) differ slightly in emphasizing the thromboplastic nature of the accessory factor, and suggest that it may be a co-factor of thromboplastin. Nolf (1908, 1938, 1945) since 1908, has maintained that there were five factors participating in human blood coagulation. His experiments were not given the recognition which they may have deserved because they lacked the excellent presentation which is characteristic of some of the workers in this field today. His five factors seem quite inadequate to explain some of the findings of other workers. A serum factor is now recognized which has been named by various investigators according to their concept of how it best fits with their interpretation of the coagulation mechanism. Owren (1947) postulated factor VI, Ware and Seegers (1947) serum accelerator globulin, Jacox (1949) prothrombin-converting factor, de Vries *et al.* (1949) serum prothrombin conversion accelerator (SPCA). Experiments were undertaken in an attempt to ascertain the relationship of thrombin A and thrombin B to the above factors.

TABLE 3.

Prothrombin and fibrinogen B formation.

	Fibrinogen B test
(1) 0·2 ml. Prothrombin + 0·2 ml. platelet suspension.	
(2) 0·2 ml. Prothrombin + 0·2 ml. M/10 Ca Cl_2.	
(3) 0·2 ml. Prothrombin + 0·2 ml. thromboplastin.	
(4) 0·2 ml. Calcium phosphate plasma + 0·2 ml. platelet suspension.	Negative
(5) 0·2 ml. Calcium phosphate plasma + 0·2 ml. M/50 Ca Cl_2.	Negative
(6) 0·2 ml. Calcium phosphate plasma + 0·2 ml. M/50 Ca Cl_2 + 0·2 ml. platelet suspension.	Negative
(7) 0·2 ml. Calcium phosphate plasma + 0·2 ml. M/50 Ca Cl_2 + 0·2 ml. thromboplastin.	Negative
(8) 0·2 ml. Calcium phosphate plasma.	Negative
Mixtures.	
(1) + (4)	Clot
(1) + (5)	++
(1) + (6)	+++
(1) + (7)	+++
(2) + (4)	Trace
(2) + (5)	Negative
(2) + (6)	Negative
(2) + (7)	Negative
(3) + (4)	Negative
(3) + (5)	Negative
(3) + (6)	Negative
(3) + (7)	Negative

The prothrombin adsorption method of Quick and Stefanini (1949) was found to be a convenient way to investigate whether or not the two components of thrombin originated from the separate precursors, accessory factor and prothrombin. In confirmation of Quick's work we have found that tricalcium phosphate will remove prothrombin, and that full activity can be restored by use of the dialysed citrate eluate. The resultant calcium phosphate plasma from the adsorption with tricalcium phosphate should contain a high concentration of all the clotting factors normally present, except prothrombin. The prothrombin from this plasma can be obtained in a relatively pure form, as it has not been subjected to any severe treatment or precipitation. The rôle of calcium ions in any mechanism which would produce or clot fibrinogen B was investigated first. Calcium phosphate plasma which contained no fibrinogen B was recalcified with an equal volume of calcium chloride to give the optimal concentration of calcium ions required for normal clotting. After incubation at 37° C. for one hour, the mixtures were tested for fibrinogen B with consistently negative results. It was also found that the addition of thromboplastin and platelet suspensions to the calcium phosphate plasma both with and without an optimal concentration of calcium ions gave negative fibrinogen B results (Table 3). It was found, however, that fibrinogen B can be formed in calcium phosphate plasma if prothrombin is re-added.

When prothrombin is re-added to calcium phosphate plasma, concentrations of calcium ions lower than the optimal for clotting will produce fibrinogen B without clotting the plasma. (See Table 4.) This may be due to the formation of small amounts of thrombin as the addition of very low concentrations of thrombin has the same effect.

TABLE 4.

Effect of Ca++ upon fibrinogen B formation in the presence of prothrombin.

0·5 ml. Calcium phosphate plasma + 0·5 ml. M/50 $CaCl_2$. + 0·2 ml. prothrombin.	Clot in about 1 hour
0·5 ml. Calcium phosphate plasma + 0·5 ml. M/60 $CaCl_2$. + 0·2 ml. prothrombin.	Clot in 1½ hours
0·5 ml. Calcium phosphate plasma + 0·5 ml. M/70 $CaCl_2$. + 0·2 ml. prothrombin.	No clot Fibrinogen B+++
0·5 ml. Calcium phosphate plasma + 0·5 ml. M/80 $CaCl_2$. + 0·2 ml. prothrombin.	Fibrinogen B++
0·5 ml. Calcium phosphate plasma + 0·5 ml. M/90 $CaCl_2$. + 0·2 ml. prothrombin.	Fibrinogen B Trace
0·5 ml. Calcium phosphate plasma + 0·5 ml. M/100 $CaCl_2$. + 0·2 ml. prothrombin.	Fibrinogen B Negative

Note: The calcium phosphate plasma was obtained from blood which had been centrifuged at 6,000 r.p.m. for 60 minutes in order to decrease the number of platelets. After centrifuging the supernatant plasma was carefully pipetted off to within one inch of the erythrocytes.

Calcium phosphate plasma was next used in order to determine whether thrombin B could be produced by the addition of platelets or thromboplastin in the presence of an optimal concentration of calcium ions. For this experiment the calcium phosphate plasma was prepared from a patient who had been exhibiting a fibrinogen B+++ in the blood continually for several days. After this plasma had been adsorbed with tricalcium phosphate, it was again tested and found to be fibrinogen B+++. The results of this investigation are set out in Table 5. It was found that fibrinogen B+++ plasma was not clotted by any combination of coagulation factors if either prothrombin or calcium ions were absent. The same experiment

was repeated using calcium phosphate plasma which was obtained from a normal person (no fibrinogen B). No clots were formed, but a mixture of prothrombin+ platelets added to this plasma in the absence of calcium ions produced a fibrinogen B+++ plasma.

TABLE 5.

Thrombin B not formed in calcium phosphate plasma.

	Clotting time	Fibrinogen B
1. Fibrinogen B+++ calcium phosphate plasma + platelets + saline.	No clot	+++
2. Fibrinogen B+++ calcium phosphate plasma + platelets + M/50 Ca Cl_2.	No clot	+++
3. Fibrinogen B+++ calcium phosphate plasma + platelets + prothrombin.	No clot	+++
4. Fibrinogen B+++ calcium phosphate plasma + platelets + saline.	No clot	+++
5. Normal calcium phosphate plasma + platelets + saline.	No clot	Negative
6. Normal calcium phosphate plasma + platelets + M/50 Ca Cl_2.	No clot	Negative
7. Normal calcium phosphate plasma + platelets + prothrombin.	No clot	+++
8. Normal calcium phosphate plasma + saline.	No clot	Negative

All constituents were mixed in equal volumes and incubated for 30 minutes at 37° C.

In reviewing Tables 4 and 5 it seems apparent that neither thrombin A nor thrombin B is derived from the accessory factor, but both are formed when prothrombin is included in the incubation mixture. This is in accordance with the experiments of Seegers *et al.* (1950) who reports that a purified prothrombin can be activated by 30 p.c. sodium citrate, and that upon resolution by electrophoresis, three components of thrombin may be present. The details of any separation of these components were not published, although Seegers suggests that one of the proteins may be an impurity carried over from the prothrombin preparation.

DISCUSSION.

Since the second stage in the conversion of fibrinogen to fibrin can be achieved by the use of organic chemicals, it seems that too little attention has been paid to the importance of the first part of this transformation which results in the formation of fibrinogen B. It is suggested that as clotting of fibrinogen cannot occur unless fibrinogen B is formed, any means to prevent the formation of fibrinogen B would necessarily prevent clotting.

Dunn, Jackson and Lyons (1949) in a survey have shown that fibrinogen B is relatively common in certain diseases, and that it rarely occurs in the normal healthy person. In view of this, the importance of this altered form of fibrinogen in pathogenic states may be of significance in other ways than as a precursor of the fibrin clot. In this investigation, the author has mainly been concerned with the production of fibrinogen B in normal human blood by the use of isolated

preparations of the factors which are already known to play a rôle in coagulation. It appeared important to discover whether thrombin A or thrombin B is derived from either accessory factor or prothrombin or whether they each had separate precursors. The results published here indicate that both originate from prothrombin. This is particularly interesting in the light of Seeger's (1950) recent work, which shows that prothrombin can be activated by certain organic chemicals and concentrated sodium citrate solution to produce potent thrombin. It is also interesting to note that electrophoretic studies on bovine thrombin indicate the presence of three components in purified specimens prepared in this manner, but he suggests that one protein is very likely devoid of thrombic activity. Also "results of electrophoresis experiments with purified thrombin preparations indicate that thrombic activity is probably associated with two proteins".

Perhaps even more interesting is the report of Shulman *et al.* (1951a, b) that the ultracentrifuge reveals a well defined intermediate between fibrinogen and fibrin when the clotting of fibrinogen by thrombin is inhibited by hexamethylene glycol.

Although efforts to combat the hypercoagulability in blood are mostly centred round the prothrombin ——→ thrombin conversion, the possibility of inhibiting coagulation and thrombosis through the separate components of thrombin, or through the two stages of the fibrinogen-fibrin reaction deserves more than academic interest.

SUMMARY.

Two components of thrombin have been isolated and their mode of action described.

Both these components appear to derive from prothrombin, and not from other accessory factors associated with coagulation.

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REFERENCES.

Chargaff, E. and Bendich, A. (1943): J. biol. Chem., 149, p. 93.
Chargaff, E. and Ziff, M. (1941): *Ibid.*, 138, p. 787.
Cummine, H. and Lyons, R. N. (1948): Brit. J. Surg., 35, p. 337.
de Vries, A., Alexander, B. and Goldstein, R. (1949): Blood, 4, p. 247.
Dunn, D. B., Jackson, M. A. and Lyons, R. N. (1949): Med. J. Austral., February 26th, p. 266.
Fantl, P. and Nance, M. H. (1946): Nature, 158, p. 708.
Feissly (1945): Schweiz med. Wschr., 75, p. 696.
Honorato, R. (1947): Amer. J. Physiol., 150, 3, p. 381.
Horan, F. E., Hirsch, F. G., Wood, L. A. and Wright, I. S. (1950): J. clin. Invest., 29, 2, p. 202–211.
Jacox, R. F. (1949): J. clin. Invest., 28, p. 492.
Jaques, L. B., Fidlar, E., Felstead, F. T. and MacDonald, A. G. (1946): Can. med. Assoc. J., 55, p. 26.
Jenner, R. (1947): Experientia, 3, p. 6.
Laki, K. and Mommaerts, W. F. H. M. (1945): Nature, 156, p. 664.
Lyons, R. N. (1945a): Austral. J. exp. Biol., 23, p. 131.
Lyons, R. N. (1945b): Nature, 155, p. 633.
Munro, F. L., Hart, E. R., Munro, M. P. and Walkling, A. A. (1945): Amer. J. Physiol., 145, p. 206.
Nolf, P. (1908): Arch. internat. Physiol., 6, p. 1.
Nolf, P. (1938): Medicine, 17, p. 381.
Nolf, P. (1945): Arch. internat. Pharmacodynam. Therap., 71, p. 1.
Oneal, W. J. and Lam, C. R. (1945): Amer. J. med. Sci., 210, p. 181.
Owren, P. A. (1947, Oslo): "The Coagulation of Blood". J. Chr. Gundersen (Oslo).
Quick, A. J. (1942): "The Haemorrhagic Diseases". Charles C. Thomas, Springfield, p. 312.
Quick, A. J. (1943): Amer. J. Physiol., 140, p. 212.
Quick, A. J. and Stefanini, M. (1948): J. lab. clin. Med., 33, p. 819.
Quick, A. J. and Stefanini, M. (July, 1949): *Ibid.*, 34, p. 973–82.
Seegers, W. H., McClaughry, and Andrews, E. B. (1950): Proc. Soc. exp. Biol. Med., 75, p. 714.
Seegers, W. H., McClaughry, R. I. and Fahey, J. L. (1950): Blood, 5, No. 5. p. 421–433.
Shulman, S., Ehrlich, P. and Ferry, J. D. (1951a): J. Amer. chem. Soc., 73, p. 1,388.
Shulman, S. and Ferry, J. D. (1951b): J. phys. coll. Chem., 55, p. 135.
Ware, A. G., Murphy, R. C. and Seegers, W. H. (1947): Science, 106, p. 618.
Zondek, B. and Finkelstein, M. (1945): Proc. Soc. exp. Biol., N.Y., 60, p. 374.

SOME DEVELOPMENTS IN RABBIT EAR CHAMBER TECHNIQUES

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The technique of the observation of a thin film of living tissue in a transparent chamber inserted into the rabbit's ear has now been in use for a number of years (Sandison, 1924, 1928; Clark *et al.*, 1930; Ebert, Florey and Pullinger, 1939; Ahern, Barclay and Ebert, 1949) and has been adapted to the mouse by Algire (1943) and Algire and Legallais (1949) and to the dog's ear by Moore (1936).

Although the principle of the method is thus well known, the technique in general is an exacting and time-consuming one, and there are many accessory pieces of apparatus which experience has shown to assist materially in the work. For that reason it may be useful to describe some of the equipment in use in this laboratory.

Design of the Chambers.

Basic design.

The basic design is a circular chamber of diameter 33 mm., with three 10 BA brass bolts, and a central table 1·5 mm. in height. The table has a bevelled edge and measures 6 mm. in diameter at the base and 5 mm. across the top, the latter being the area of tissue available for observation. It has been found that bevelling the edge facilitates somewhat the rate of organization of the area of the table-top. The whole chamber is turned out of $\frac{1}{8}$ inch perspex sheet, and the optical properties of the top surface of the table are best preserved by leaving on that surface during machining the paper shield provided by the manufacturer. The four small perspex "buffers" are cemented to this surface with shellac in alcohol in the usual way. In general they should be 40μ–50μ in thickness. Thinner films of tissue are very slow to organize, and in them organization is in fact very often incomplete, a mass of debris being left unorganized in the centre, surrounded by a palisade of fibrous granulation tissue. In the course of time this becomes less vascular and more fibrous in character. On the other hand, very thick films, though they organize very rapidly, are obviously much less satisfactory for microscopic observation with higher powers.

The bolts are turned out of brass rod and project 10 mm. above and below the base. The securing hexagonal nut which rests on the upper surface of the base is turned down until it is 1·4 mm. in thickness. It thus forms a convenient washer as well.

The *lid* in its simplest form is a ring of perspex 33 mm. in outside diameter, 20 mm. in inside diameter, with a beveiled upper inner edge, on to the under surface of which is cemented

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a coverslip of glass, mica or perspex. Mica coverslips have been most used, as they combine strength with thinness, but they introduce difficulties when working with polarized light. Glass coverslips are apt to break under the constant bending stress to which they are exposed when the lid is screwed down on to the buffers. Most workers seem to have used Varian's (1931) cement to fix the coverslip to the ring. Though very suitable this substance is rather troublesome to prepare, and it has been found best to regard lids as expendable items, and to use the universally available Canada balsam as the cement. Care must be taken in applying it to clean off any excess which exudes around the edge of the coverslip during application, as this tends to provoke tissue reaction and so interferes with the vascularization of the chamber. A further protection is afforded by machining the undersurface of the lid so that the coverslip is slightly inset.

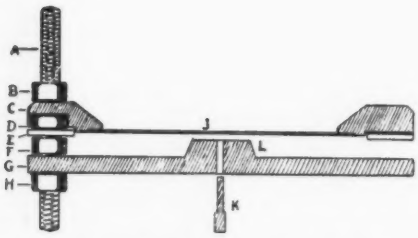

Fig. 1. Section of chamber with removable lid.

A. 10 BA bolt (brass).
B. 10 BA nut (brass).
C. Perspex lid recessed to take D and E., 2·5 mm. thick × 6·5 mm.
D. 10 BA nut (brass) turned to 1 mm. thick.
E. 0·5 mm. aluminium retaining ring 4 mm. across.
F. 10 BA nut (brass) turned to 1·4 mm. thick, acting as spacer for ring E.
G. Base of chamber 1·5 × 33 mm.
H. 10·BA nut (brass).
J. Mica or perspex coverslip.
K. Perspex pin. Shank 0·4 mm. thick × 3 mm. long.
L. Table 1·5 mm. high × base diameter 6 mm; face diameter 5 mm.

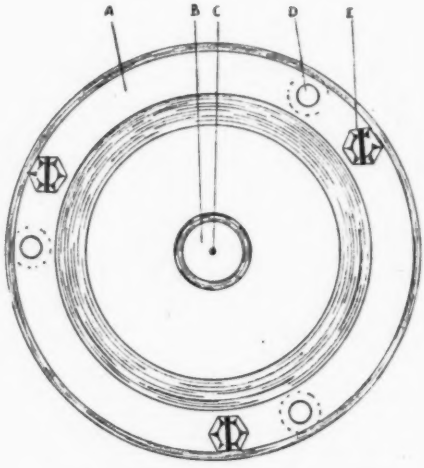

Fig. 2. Plan of chamber with removable lid.

A. Perspex lid.
B. Central table.
C. Access pin.
D. Hole to take 10 BA bolt and recessed on underside for D of Fig. 1.
E. 10 BA bolt with screw slot or milled head for lifting lid.

Sanders (1950) has devised a removable lid, which may be lifted and replaced, should it be deemed necessary by some circumstance such as infection of the chamber, without disturbing the anchorage of the base of the chamber in the ear. In this manner it may be possible to save a preparation without the necessity of completely removing and re-inserting the chamber. This lid (Fig. 1) has been slightly simplified by using a light metal (aluminium) retaining ring, instead of a perspex ring. By this means the ring is made more rigid without materially increasing its weight, and it is then possible to put the threaded lifting holes close to the bolt holes (Fig. 2), and to lift the lid gradually merely by turning milled-headed

10 BA screws inserted into the lifting holes and bearing on the metal ring. Sterile saline is meanwhile run in through a hypodermic needle inserted under the edge of the lid. Intravenous injection of 50,000 units of penicillin, together with 0·075 gm. of streptomycin, immediately before inserting the chamber, materially reduces the incidence of subsequent infection.

Access chambers.

The simplest type of access chamber is that which has a central pin of silver, brass or perspex passing through the base and reaching exactly to the level of the top of the table, or, in some cases, to the under surface of the coverslip if it is not desired to permit the tissue to organize across the top of the pin.

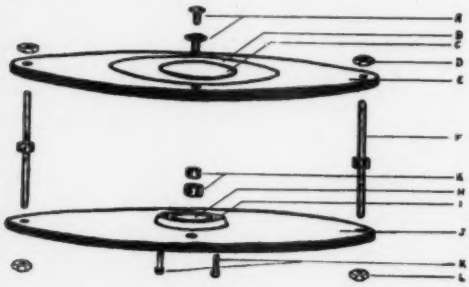

Fig. 3. Large oval chamber with quartz table and coverslip.

A. 10 BA bolts with flat slotted heads.
B. Recess in lid for quartz cover.
C. Quartz cover.
D. Lock nut, 10 BA. Turned to 1 mm. thick.
E. Perspex lid.
F. 10 BA bolt threaded each end, with shoulder in centre to act as washer.
G. Perspex washers drilled and tapped 10 BA, to be cemented to base.
H. Quartz table top, diameter 6 mm.
I. Perspex table 1·5 mm. high, drilled 5·5 mm. and recessed to take quartz disc.
J. Perspex base, 47 mm. long axis × 33 mm. short axis.
K. Perspex pins, approx. 0·4 mm. diameter × 3 mm. shank.
L. Lock nut, 10 BA. Turned to 1 mm. thick.

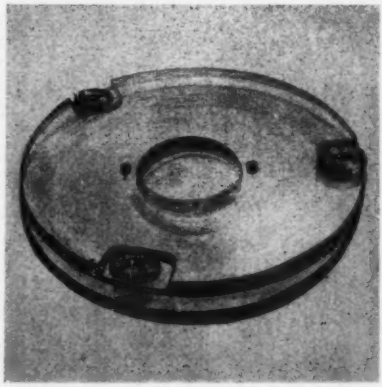

Fig. 4. Thin circular access chamber with quartz table and cover.

Perspex pins provoke the least reaction and are naturally much superior in optical properties. A diameter of about 0·4 mm. is possible for a pin of 3 mm. length if a jeweller's lathe is used and the work revolved at very high speed during machining. Although Ahern, Barclay and Ebert (1949) found it best to use loosely fitting pins, this has not been found to be necessary if the procedure to be described below for the removal of the pin is followed.

A useful modification is to place the pin *eccentrically* in the table. If this is done much less time is taken for the tissue to organize over the top of the pin (organization always proceeds much more slowly near the centre of the table) so that the chamber may be used for implantation rather earlier than would be the case with a central pin. Extreme care must,

however, be used in withdrawing the pin in such cases, as the very newly organized tissue is much more delicate than tissue of longer standing. A further advantage of an eccentric pin is that it may often be useful to use a chamber with more than one pin, which enables the introduction of two or more substances or tissues.

Further special types of access chambers have been developed for work involving observations with reflecting objectives in ultraviolet light. These objectives are of much larger diameter than the conventional refracting type, and this considerably complicates the design of the chamber, as, if the chamber is of a conventional type, the objective has during examination, to pass down through the circle of bolts holding the chamber together, and more than this, must be capable of at least enough traverse for observations to cover the area of the central table. The diameter of the face of the objective is 35 mm. Therefore the circle on which the chamber bolts are placed must be a large one.

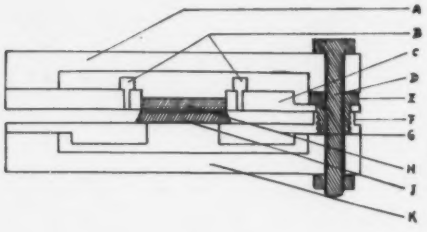

Fig. 5.

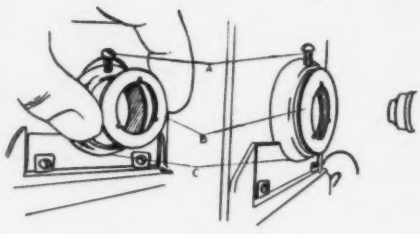

Fig. 6.

Fig. 5. Thin circular access chamber with quartz table and cover (section).

A. Perspex shield.
B. Perspex pins. Shank 1·6 mm. × 0·46 mm. diameter.
C. Perspex lid diameter 27 mm. with 6·5 mm. hole in centre for quartz disc.
D. 10 BA bolt and nut for holding shields.
E. 7 BA screw with 2·25 mm. shank drilled to take 10 BA bolt.
F. Perspex washer 0·96 mm. high, threaded 7 BA and cemented to base.
G. Perspex base diameter 27 mm. with 6 mm. hole in centre.
H. Quartz disc 6·5 mm. diameter × 1 mm. inset in lid.
J. Quartz disc 6·5 mm. diameter × 1 mm. cemented over hole in base.
K. Perspex lid.

Fig. 6. Dome and ring for locking ear chamber to stage.

A. Lock-screw.
B. Retaining ring (to be bolted to base of chamber).
C. Stage dome to take B.

As there would be grave anatomical difficulties in introducing a large circular chamber into the ear of a rabbit, an oval chamber (Fig. 3) has been designed which is inserted with its long axis in the long axis of the ear. The chamber has 4 bolts, of which those on the long axis project on both the upper and lower surface of the chamber, while those on the short axis are short flat-topped screws which are screwed into the base from the top. There are therefore only two bolts for the objective to clear, and they are 47 mm. apart.

The stage of this chamber is formed of a quartz circle 6 mm. in diameter and 0·8 mm. in thickness inset into a circular perspex stage 11 mm. in diameter. The quartz disc is cut from a microscope slide. Two perspex access pins enter the edge of the table on opposite sides,

through the perspex boundary from below, the buffers being cemented to the surface of the quartz. The coverslip is formed by a larger circle of quartz of similar thickness set in the oval perspex lid of the chamber. By this means it is possible to introduce fluid substances through the pinholes, and some of the substance diffuses into the organized tissue on the quartz table where it is accessible to observation in ultraviolet light.

It would naturally be more suitable to have a thin quartz pin passing through a cylindrical hole drilled through the quartz table, but this would be difficult to achieve and has not been attempted.

A further type of access chamber with quartz optical parts is shown in Figs. 4 and 5. This is superior to the design shown in Fig. 3 as it is much smaller, and has a central table entirely of quartz, without a large surrounding rim of perspex. The absence of the latter naturally makes for much quicker organization as the area to be covered is reduced by more than 50 p.c. This design has been made possible by placing the access pins in the lid, and by using very short *hollow* bolts (No. 7 BA), through which pass removable 10 BA bolts serving to secure the shields. The chamber is thus thin enough to fit in its entirety between the reflecting objective and condenser. The bolts are inset 1 mm. both in the base and the lid, to reduce still further the thickness of the chamber, and are screwed into perspex washers cemented to the base, and of a height adjusted to be equal to or very slightly less than the height of the central quartz table.

To facilitate insertion of the three short hollow bolts, each of the latter is slipped over a guide rod threaded 10 BA at one end, and secured to it by a 10 BA nut. The end of the rod is then passed through the lid and through the perspex washer on the base. The bolt is then readily entered by a twist of the fingers, and the rod is removed. The 7 BA screws are tightened further with a small screwdriver.

The two perspex access pins, of diameter 0·46 mm., pass through the lid one on each side of the quartz coverslip, which is the same size and thickness as the table, and is set flush with the under surface of the perspex lid. The 4 buffers are cemented to the under surface of the lid. The 6·5 mm. quartz disc forming the table is centred over a 6 mm. hole in the centre of the base, and sealed, as is the coverslip, by running thin perspex in chloroform carefully around it.

Accessory Equipment.

Optical bench assembly.

A horizontal microscope is used mounted on a parallel-railed optical bench. A shallow brass dome or ring is mounted on the stage of the microscope (Fig. 6C). To observe the tissue in a chamber, a brass adapting ring (Fig. 6B) is screwed to the under surface of the base of the chamber by means of the chamber bolts after removing the aluminium shield, and the ear being brought up to the stage, this ring is slipped into the dome where it is at once firmly secured by tightening the screw (Fig. 6A) passing through the dome. A shallow groove into which the point of the screw enters, on the outer surface of the adapting ring, prevents any possibility of slipping. As chambers of different sizes are in use the dome is made to accommodate the largest, and smaller chambers are adapted to it by special rings perforated to accommodate the bolts of the chamber in question.

The removal of a pin for implantation of material.

For the removal of the pin from an access chamber, a pin vice is screwed into the objective holder of the microscope in place of one of the objectives (Fig. 7). The brass adapting ring is screwed to the upper surface of the chamber, so that when mounted in the dome the base of the chamber is uppermost and the pin projects. After carefully cleansing the area

around the base of the pin the pin vice is lowered to grip the pin. A wide perspex collar on the vice makes tightening much easier without obscuring vision of the area. The vice and pin are then carefully and slowly rotated through approximately 90° to break the seal of serum which holds the pin in the hole. The pin is then withdrawn by using the fine adjustment of the microscope, the whole operation being watched from the top by a dissecting microscope or binocular loupe, and from below by a modification of the microscope which permits observation from this aspect.

This modification consists in mounting the body tube of the microscope in a bracket (Fig. 7H) fitted permanently to the under surface of the stage. The body tube bears a 90° prism permitting observation up through the condenser diaphragm, while a 16 mm. objective is mounted in the condenser fitting after removing the condenser. It is focussed by use of the condenser rack and pinion. Illumination is provided by a beam passing down through the centre of the hollow pin vice and through the perspex pin. By this means the operator can be sure that trauma does not occur in the film of tissue during the removal of the pin.

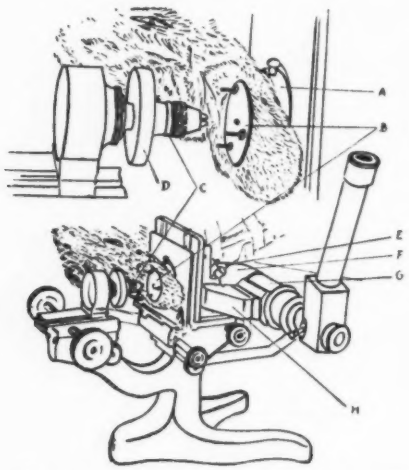

Fig. 7. Equipment for removal of access pin.

A. Stage dome.
B. Chamber.
C. Pin vice.
D. Perspex ring on C enabling easy manipulation and clear vision.
E. 90° prism.
F. Prism mount.
G. Monocular body tube with watching eyepiece.
H. Substage bracket mount for G.

Illuminants.

For routine observation a 6 volt 100 watt coiled coil projector lamp is used, mounted on the optical bench and provided with the usual condensers and a water cell. The latter is important as the film of tissue is most susceptible to heat. A Photoflood lamp can be substituted for photography in colour, although the 6 volt lamp has a colour temperature almost adequate for kodachrome work. The ultraviolet lamp is a Hanovia type mercury discharge tube, and the 2,536 Å and 2,650 Å lines are isolated by use of the liquid filters advocated by Bowen (1942). A photocell and amplifying system has been found most useful for exposure determinations, and has even been adapted as a microphotometer.

Other equipment.

Protecting shields for the chamber. A simplification of the technique previously described by Ebert, Florey and Pullinger (1939) is the use of protecting shields mounted directly on to the chamber bolts. This enables the shields to be kept small and light, and does away with the necessity for special external splints. Aluminium or perspex is the most suitable material for the shields.

Animal table. As it is often necessary to posture the rabbit carefully in order to get the ear into position on the stage, a special table which can be rotated about its long axis is an advantage, the rotation being possible through a considerable range, and without loosening any of the restraining bands which hold the animal to the table.

SUMMARY.

Some developments in rabbit ear chamber techniques are described. These include two special types of access chamber with quartz optical parts for observations in ultraviolet light, together with equipment aiding the implantation of materials and the observation of the chambers.

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REFERENCES.

Abern, J. J., Barclay, W. R. and Ebert, R. H. (1949): Science, 110, p. 665.
Algire, G. H. (1943): J. Nat. Cancer Inst., 4, p. 1.
Algire, G. H., Legallais, F. Y. (1949): *Ibid.*, 10, pp. 225–53.
Bowen, E. J. (1942:) "The chemical aspects of Light." Oxford Clarendon Press.
Clark, E. R., Kirby Smith, H. T., Rex, R. O, and Williams, R. G. (1930): Anat. Rec., 47, p. 187.
Ebert, R. H., Florey, H. W. and Pullinger, B. D. (1939): J. Path Bact., 48, p. 79.
Moore, R. L. (1936): Anat. Rec., 64, p. 387.
Sanders, A. G. (1950): Personal Communication.
Sandison, J. C. (1924): Anat. Rec., 28, p. 281.
Sandison, J. C. (1928): Amer. J. Anat., 41, p. 447.
Varian, B. (1931): Science, 73, p. 678.